Thesis submitted for the degree of

Doctor of Philosophy

Defective circulating and intrahepatic B cells in chronic HBV

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"We marvel at the details but we must also pan out to the big picture, because it's only when we do this that we can exploit our knowledge of immunity for a revolution in health"

Daniel M. Davies, A Beautiful Cure

Declaration

I, **Alice Roselyn Burton**, confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in the thesis.

Signature: _____

Date: _____

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Abstract

B cells are increasingly recognised to play an important role in the ongoing control of chronic viral infections, yet their contribution to the persistence of HBV is poorly understood. The development of antibodies against HBV envelope protein (HBsAg) is considered the hallmark of resolution of acute infection; accordingly, seroconversion against HBsAg represents a key therapeutic goal for functional cure of chronic HBV (CHB). The production of inadequate anti-HBsAg (anti-HBs) antibodies to neutralise large quantities of circulating HBsAg in chronic infection suggests there may be defects in the B cell response upon HBV infection. To investigate constraints on their antiviral potential, global and antigen-specific B cells were characterised directly *ex vivo* from the blood and liver of patients with CHB. HBsAg-specific B cells persisted in the blood and liver in patients with CHB at similar frequencies to patients with acute-resolving infection and healthy HBV-vaccinated controls. However, differentiation of HBsAg-specific B cells from patients with CHB demonstrated decreased antibody production, consistent with undetectable anti-HBs antibodies *in vivo*. HBsAg-specific and global B cell compartments had an accumulation of CD27^{-}CD21$^{low/-}$ atypical memory B cells (atMBCs), characterised by high expression of inhibitory receptors, including PD-1. These atMBCs demonstrated altered signalling, homing, differentiation into antibody-producing cells, survival and antiviral cytokine production, that could be partially rescued by PD-1 blockade. PD-1^{hi} atMBCs were further expanded in healthy and HBV-infected livers compared to the periphery, implicating the combination of this tolerogenic niche and HBV infection in driving PD-1^{hi} atMBCs and impairing B cell immunity. Finally, the identification of endogenous antigen-specific B cells demonstrates previously unappreciated potential for these cells in natural control of HBV that may be enhanced through immunotherapeutic targeting. Work presented in this thesis has start to identify targets for reconstitution of their antiviral efficacy.

Impact Statement

Recent studies have highlighted the increasing burden of disease attributable to hepatitis B virus infection. In chronic HBV infection (CHB), the immune response fails to control the virus and instead triggers tissue damage, culminating in an estimated ~700 000 deaths a year worldwide. Current treatment approaches fail to induce off-treatment viral control, highlighting a pressing need to develop new and improved therapeutic strategies. The success of immunotherapy for the treatment of cancer, highlighted by the recent Nobel Prize awarded for the discovery of immune checkpoints, shows significant promise in the development of new treatment regimens for the control of chronic viral infections, that are well-tolerated by patients and able to maintain long-term viral control.

CHB is characterised by exhausted adaptive immunity in association with an absence of detectable protective antibodies against the HBV envelope protein (HBsAg). While much progress has been made in identifying targets underpinning T cell exhaustion in the setting of persistent antigenic stimulation, detailed characterisation of anti-HBV-specific B cell responses is lacking. Through analysing the frequency, function and phenotype of global and HBsAg-specific B cells, we show that HBsAg-specific B cells can persist in the blood and liver of patients with CHB. However, both global and antigen-specific B cell compartments are enriched for B cells with a dysfunctional, atypical memory phenotype, characterised by increased expression of co-inhibitory molecules, including PD-1. In characterising these cells, this study has begun to identify targets for the immunotherapeutic boosting of B cell immunity, which may have important implications for the design of therapeutic vaccines and other approaches aiming to induce co-ordinated immune responses.

In particular, blockade of checkpoint molecules, such as PD-1, represents a key immunotherapeutic approach envisaged to restore exhausted adaptive immune responses and rescue control of viral infection; as such, anti-PD-1 therapy, in combination with therapeutic vaccination, is now undergoing testing in patients with CHB. In order to maximise efficacy and minimise off-target effects, it is important to understand how PD-1-targeted therapy affects all aspects of the immune response. In this respect, we show that PD-1 blockade can modestly increase pro-inflammatory cytokine production by B cells, but may potentiate the accumulation of dysfunctional B cell subsets. This recognition that humoral responses may be affected by PD-1 targeted therapy may therefore have important implications for the application of checkpoint blockade in patients with CHB.

Finally, the concept of persistent antigen stimulation perturbing memory B cell responses has been gathering impetus over recent years, with many papers now demonstrating hypo-responsiveness of memory B cells in settings of chronic infection, autoimmunity and ageing. Natural infection with HBV provides an ideal setting to compare the effects of persistent and resolving infection with vaccine-induced immunity on B cell subsets. This study is also one of the first to describe antigen-specific memory B cells in a non-lymphoid organ, at the site of viral infection. Thus, these data may have broader impmlications for understanding the role of B cells within non-lymphoid tissues.

List of Publications

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European Association for the Study of Liver (2017) – Poster presentation. Also selected for oral e-presentation and expert-guided poster tours (HBV virology and HBV immunology session)$^{\delta}$

BSI: Chronic diseases and infection (2018) – Oral presentation

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List of Abbreviations

Ab	antibody
actMBC	activated memory B cell
ADCC	antibody-dependent cellular cytotoxicity
ADCP	antibody-dependent cellular phagocytosis
AID	activation induced deaminase
ALF	acute liver failure
ALT	alanine transaminase
anti-HBs	anti-HBsAg antibodies
anti-HBc	anti-HBcAg antibodies
APC	antigen presenting cell
ASC	antibody secreting cell
atMBC	atypical memory B cell
BALT	bronchus-associated lymphoid tissue
Bcl-2	B cell lymphoma 2
BCR	B cell receptor
BIM	Bcl-2-interacting mediator of cell death
BLNK	B cell linker protein
bnAb	broadly neutralising antibody
Breg	B regulatory cell
BTLA	B and T lymphocyte attenuator
cccDNA	covalently closed circular DNA
CCL	CC chemokine ligand
CD	cluster of differentiation
CHB	chronic hepatitis B
cMBC	classical memory B cell
CMV	cytomegalovirus
CRC	colorectal cancer
CTLA-4	cytotoxic T-lymphocyte-associated protein 4
CVID	combined variable immune deficiency
CXCL	CXC chemokine ligand
CXCR	CXC chemokine receptor
DMSO	dimethylsulfoxide
DNA	deoxyribose nucleic acid
EAE	experimental autoimmune encephalomyelitis
EASL	European Association for the Study of the Liver
EBV	Epstein Barr Virus
EDTA	ethylenediaminetetraacetic acid
ELISA	enzyme-linked immunosorbent assay
ELISPOT	enzyme-linked immunospot
ERK	extracellular signal-regulated kinase
FBS	fetal bovine serum
Fab	fragment antigen-binding
FACs	Fluorescence-activated cell sorting
Fc	fragment crystallisable
FcR	Fc-receptor
FcRL4/5	Fc-receptor like 4/5
FDC	follicular dendritic cell
GC	germinal centre
HBcAg	hepatitis B core antigen
HBeAg	hepatitis B e antigen
HBsAg	hepatitis B surface antigen
HBV	hepatitis B virus
HBx	hepatitis B x protein
HCC	hepatocellular carcinoma
HCV	hepatitis C virus
HLA	human leukocyte antigen
HRP	horseradish peroxidase
HSPG	heparan sulphate proteoglycan
IC	immune complex
IFN	interferon
IHL	intrahepatic lymphocytes
Ig	immunoglobulin
IL	interleukin
ILF	intrahepatic lymphoid follicle
IU	international units
KC	Kupffer cell
LAG3	Lymphocyte activation gene 3
LPS	lipopolysaccharide
LSEC	liver sinusoidal endothelial cell

mAb	monoclonal antibody
MAIT	mucosal-associated invariant T cell
(g)MDSC	(granulocytic) myeloid derived suppressor cell
MHC	major histocompatibility complex
MFI	mean fluorescence intensity
mRNA	messenger RNA
nAb	neutralising antibody
NFAT	nuclear factor of activated T cells
NK	natural killer
NKT	natural killer T cell
NKG2D	natural killer group 2 receptor D
NUC	nucleoside
NTCP	sodium taurocholate cotransporting peptide
OD	optical density
ORF	open reading frame
PBS	phosphate buffered saline
PD-1	programmed-death 1
PD-L	programmed-death ligand
Peg	pegylated
PI3K	phosphoinositide 3-kinase
RA	Rheumatoid arthritis
RIG	retinoic acid-inducible gene
RNA	ribose nucleic acid
RPMI	Roswell Park Memorial Institute medium
SCS	subcapsular sinus
SHM	somatic hypermutation
SIV	Simian immunodeficiency virus
SLE	Systemic lupus erythematosus
Syk	spleen tyrosine kinase
T-bet	T-box expressed in T cells
Th	T helper
T_{FH}	T follicular helper
TGF	transforming growth factor
Tim-3	T cell immunoglobulin and mucin domain 3
TLR	toll-like receptor
TNF	tumour necrosis factor
TRAIL	TNF related apoptosis-inducing ligand
TRAIL-R2	TNF related apoptosis-inducing ligand receptor 2
Treg	T regulatory cell
UCLH	University College London Hospital
WHO	World Health Organisation

Chapter 1 Introduction

1.1 The immunological revolution

The realisation that the human body is innately programmed to fight any entity it detects as foreign and dangerous came only as recently as 1989. Although many eminent scientists, including Edward Jenner, had alluded to the ability of the body to recognise *non-self*, the first description of the ability of the immune system to detect when something is likely to be a threat was conveyed in a seminal paper published by Charles Janeway (Janeway, 1989). Since then, many important discoveries have been made that piece together the intricacies of the human body to fight invading pathogens. Not only have these revolutionised the way that we view the human body, these important findings have ignited transformative approaches for medicine in the 21st century.

It is now apparent that layer upon layer of immune checks and balances regulate interactions between a diverse range of immune cell types. Together, these cells mastermind co-ordinated responses that fight infection and minimise destruction caused. Where these responses go wrong, chronic infection and tissue damage can ensue.

Already we have found ways to employ the immune system to create innovative new drugs, referred to as immunotherapies, that have shown significant promise in the treatment of cancer, arthritis and diabetes. The current excitement surrounding the power of body's own defence system has been recently exemplified by the success of popular science books such as 'The Beautiful Cure', by Dan Davies at the University of Manchester (Davis, 2018). Moreover, immunologists central to the characterisation of the immune response and the development of ground breaking immunotherapies were recently honoured at the highest level, with Professor John P. Allison and Professor Tasuku Honjo awarded the 2018 Nobel Prize in Physiology or Medicine for their discovery of cancer therapy by checkpoint blockade, and Sir Gregory P. Winter awarded the 2018 Nobel Prize in Chemistry for his work on phage display of peptides and antibodies. These examples, highlighting the importance of immunology in modern medicine, demonstrate the power and potential of immunological research, and advocate for the continued study of the multifaceted immune response following infection.

1.2 Overview of HBV

1.2.1 The global health burden of HBV

Intensive efforts over the last 20 years have succeeded in reducing mortality from communicable disease with the notable exception of viral hepatitis. Whilst deaths arising from the WHO's top three priorities – HIV-AIDs, malaria and tuberculosis - have consistently fallen, mortality and morbidity attributable to viral hepatitis have continued to rise, with the annual death rate attributable hepatitis B virus (HBV) or hepatitis C virus (HCV) infection increasing by approximately 60% over the last 20 years (Stanaway et al., 2016). Both viruses are able to establish persistent infection that can evolve to liver cirrhosis and hepatocellular carcinoma; as it stands, an estimated 240 million people are chronically infected with HBV, with HBV-related complications resulting in approximately 780,000 deaths a year worldwide (Stanaway et al., 2016). A significant proportion of HBV infection is concentrated in highly endemic areas, predominantly sub-Saharan Africa and Asia, where up to 20% of the population is infected (Liaw and Chu, 2009)

HBV transmission can occur via a number of different routes: sexual, parenteral (for example through illicit drug use or via blood transfusion products) or perinatal (from HBV-infected mothers to neonates). Transmission in highly endemic areas is largely perinatal (vertical transmission, common in East Asia) or occurs very early during childhood via horizontal transmission, for example via saliva and open wounds. Horizontal transmission most commonly occurs in households with a persistent carrier and is most readily detected in Sub-Saharan Africa (Gray Davis et al., 1989). In contrast, infection in low prevalence areas tends to be acquired during adulthood. In high-income countries, HBV prevalence is heavily influenced by global migration; for instance, the prevalence of HBV-infection in London - where this thesis was written - has been estimated at ~2.02 per 100,000 of the population, nearly twice as high as the rest of England and reflective of the multicultural demographic of the capital. [Data collected from Public Health England; www.gov.uk/government/publications/hepatitis-b-epidemiology-in-london-2012].

As with most communicable diseases, effective prevention strategies are key to reducing the burden from disease. Current available prophylactic vaccines are highly effective and protect ~94-98% of individuals from infection. Where vaccination strategies capture at-risk populations early in life, prevalence of HBsAg can be reduced to zero, resulting in concomitant decreases in HBV-related disease (Chen, 2009). However, the application of large-scale vaccination strategies can be difficult and often fails in the absence of effective public health education and deliverance. This is further complicated by the necessity of three doses required for adequate protection, which means that coverage and compliance can be low. Moreover, vaccine efficacy varies markedly between different populations, with approximately 5-10% of individuals failing to mount an effective antibody response following vaccination. These differences are thought to be a consequence of genetic predisposition and immune status (Walayat et al., 2015) and has resulted in the development of

more immunogenic hepatitis B vaccines that target protective regions of HBV and require fewer doses (discussed in more detail in section 1.6.1).

Hence, whilst effective vaccines are available, there remains a clear necessity for drugs to treat HBV-infected individuals and reduce carriage of the virus. Whilst new therapies for HCV have delivered striking results, with more than 90% of patients achieving viral clearance following treatment with directly acting antivirals (Foster et al., 2015), current treatment strategies for HBV, namely pegylated-interferon-alpha (Peg-IFNα) and nucleoside analogues (NUCs), rarely achieve functional cure (defined as sustained loss of viraemia and HBV-surface antigen (HBsAg) following termination of therapy). Since NUCs target HBV at the level of DNA synthesis only, they are unable to eradicate the episomal form of HBV and thus necessitate long-term therapy. Importantly, this persistence of episomal and integrated DNA provides an ongoing source of secreted HBsAg and thus represents a key target for new therapeutic strategies. Numerous studies are now investigating the potential for NUC-therapy withdrawal, with the aim to identify patient groups that are able to maintain viral suppression following NUC discontinuation (Berg et al., 2017; Hadziyannis et al., 2012; Rivino et al. 2018).

Alongside additional problems associated with cost, compliance, toxicity and global access, there is a pressing need to develop new strategies that are better tolerated by patients and provide ongoing immune surveillance, as is observed in resolved HBV. The recent successes of immunotherapies for treatment of cancer raises the prospect of immunotherapeutic approaches for the treatment of chronic HBV, supported by the rationale that the immune response is able to maintain long-term viral control in the majority of patients who are acutely infected with HBV (Maini and Pallett, 2018). Thus, restoration of defective cellular and humoral antiviral immunity is likely to be indispensable for off-treatment virus control. These strategies should also aim to induce neutralising antibody responses that can prevent the spread of infection from hepatocytes and thereby prevent reactivation of HBV replication (discussed in more detail in section 1.6.1).

1.2.2 HBV life cycle

HBV is a non-cytopathic, partially double-stranded DNA virus of the *Hepadnaviridae* family and can be categorised into ten distinct genotypes (A-J) based on divergence in the viral sequence. These strains differ in their geographical distribution and may correlate with disease progression and treatment response (Lin and Kao, 2015). As a hepatotropic virus, HBV can efficiently infect a large proportion of hepatocytes and establish persistent infection in the liver (Protzer et al., 2012; Seeger and Mason, 2015). Pathology arises due to a failure of the immune response to control the infection, instead triggering tissue damage leading to liver cirrhosis and cancer.

HBV is one of the smallest DNA viruses with a genome of approximately 3200 nucleotides that encodes seven viral proteins in four overlapping reading frames (Figure 1.1). The infectious HBV

virion (Dane particle) has a spherical, double-shelled structure consisting of a lipid envelope containing the envelope protein, HBsAg. This surrounds an inner nucelocapsid composed of hepatitis B core antigen (HBcAg) complexed with virally encoded polymerase and the viral DNA genome. HBV virions initiate interactions with hepatocytes through interactions with cell surface glycoproteins referred to as heparan sulphate proteoglycans (HSPGs) (Schulze et al., 2007). These proteins act as a low affinity receptor for HBV and localise virions to the liver to enhance viral binding and entry (Leistner et al., 2008). Following initial attachment to HSPGs, HBV virions bind to sodium taurocholate co-transporting peptide (NTCP) through specific interactions between NTCP and the HBV large surface protein (HBsAg) (Ni et al., 2014; Yan et al., 2012). NTCP is expressed exclusively by hepatocytes and is responsible for the transport of conjugated bile salts; thus, the ubiquitous expression of NTCP by hepatocytes is postulated to underpin the tropism of HBV for the liver.

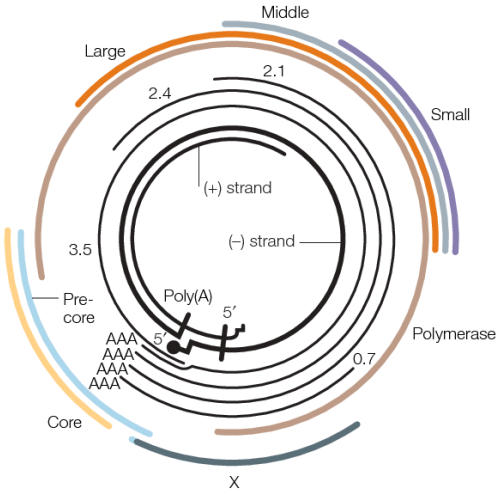

Figure 1-1 Schematic depicting the HBV genome

HBV is a partially double-stranded DNA virus. The innermost circles (black) represent the full length minus strand (-) and the incomplete plus strand (+) of the genome. The seven HBV viral proteins (discussed in 1.2.2) are shown by the outermost coloured lines: large, middle and small HBV surface proteins, polymerase protein, X protein and core and pre-core proteins. Adapted from (Rehermann and Nascimbeni, 2005).

Following viral entry, virions undergo a process of uncoating, whereby partially double stranded circular DNA (rcDNA) is delivered into the cytoplasm of the hepatocyte (Figure 1.2). HBV nucleocapsids translocate to the nucleus, where the relaxed circular DNA is transformed into covalently closed circular DNA (cccDNA). cccDNA provides a template for the transcription of viral mRNAs, driven by a host polymerase II enzyme (Pol-II). Viral RNAs are then transported to the cytoplasm where they are translated to produce HBV surface, pre-core/core, polymerase and X proteins, as follows (Beck and Nassal, 2007; Dandri and Locarnini, 2012; Grimm et al., 2011; Liang, 2009; Rehermann and Nascimbeni, 2005; Tu and Urban, 2018):

1. *Core:* Gives rise to distinct proteins, depending on whether translation is initiated from the core or pre-core regions
    a. *Pre-core protein:* transported to the endoplasmic reticulum and excreted as secretory HBeAg
    b. *Core protein:* forms the nucleocapsid protein (HBcAg) required for capsid assembly
2. *Envelope proteins:* consisting of three different sizes, small (S), middle (M) and large (L)
3. *HBx protein:* activates HBV promoter activity and required for the initiation of infection
4. *Polymerase:* reverse transcriptase required for DNA synthesis

Resultant proteins are assembled to form nucleocapsids, into which a strand of pregenomic RNA is encapsidated within the polymerase protein and reverse transcribed to minus-strand DNA. This minus-strand DNA is then used to synthesize plus-strand DNA. Resulting capsids either bud into the endoplasmic reticulum, where they are enveloped with L, M and S surface antigens and released as complete virions, or transported back to the nucleus, where they are converted to cccDNA and maintain the pool of templates (Beck and Nassal, 2007; Dandri and Locarnini, 2012; Grimm et al., 2011; Rehermann and Nascimbeni, 2005; Tong and Revill, 2016; Tu and Urban, 2018; Urban et al., 2014; Verrier et al., 2016). As a replicative intermediate, cccDNA is central to the persistence of HBV, proving an ongoing template for the transcription of viral proteins. In this way, persistence of cccDNA presents a major target in the development of future treatment regimens aiming to achieve complete virus eradication.

The replication of HBV via reverse transcription of an RNA intermediate distinguishes HBV from all other known mammalian DNA viruses. Reverse transcription is inherently error-prone and as such, incorporates errors and mutations to the genomic sequence. This process results in the emergence of highly heterogeneous viral populations, referred to as quasispecies. Such variation within populations presents a reservoir for viral selection under pressure from antiviral treatment and the immune response (Aragri et al., 2016; Cao et al., 2014).

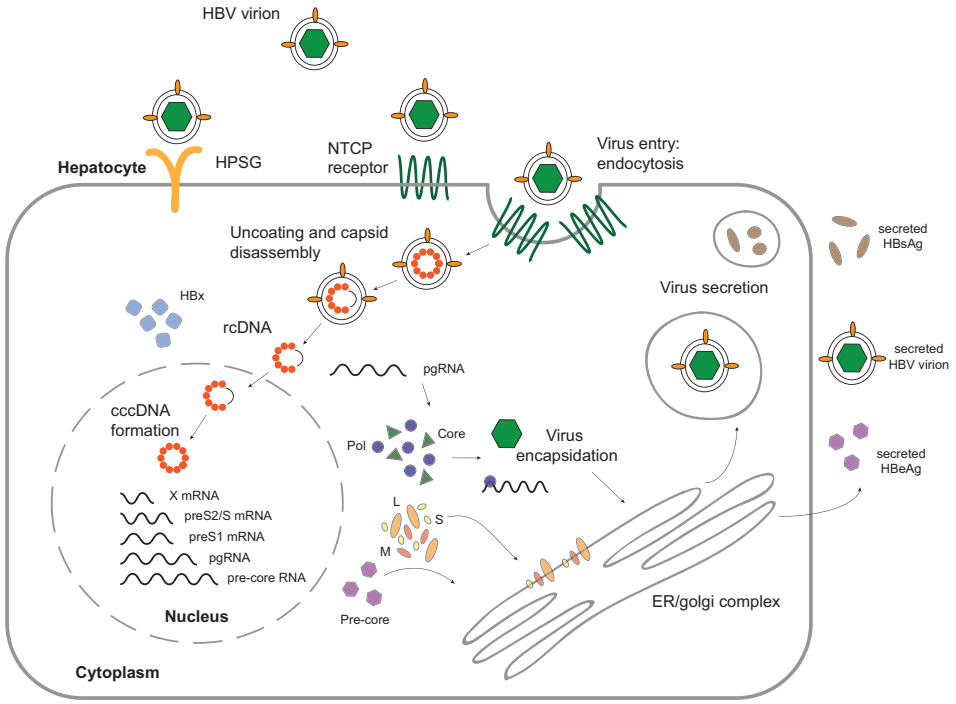

Figure 1-2 Schematic of the HBV replication cycle

Schematic depicting the life cycle of HBV following entry into the hepatocyte via NTCP. Full details of the viral life cycle are outlined in 1.2.2. [Abbreviations used: cccDNA = covalently closed circular DNA; rcDNA = relaxed, partially double stranded DNA; NTCP = sodium taurocholate co-transporting polypeptide; HSPG = heparan sulphate proteoglycans]

1.2.2.1 Hepatitis B surface antigen (HBsAg)

In addition to the secretion of whole virions, large amounts of spherical and filamentous HBsAg particles are also secreted, where they outnumber virions by a factor of 10^4-10^6 (Rehermann and Nascimbeni, 2005). HBsAg was the first identified component of HBV, discovered by Baruch Blumberg in 1965; initially named 'Australia Antigen', HBsAg was identified in the serum of haemophiliac patients and reacted against proteins present in the serum of an Aboriginal Australian (Blumberg, 1977; Blumberg and Alter, 1965). HBsAg was later demonstrated to be an envelope protein consisting of several subtypes that differ in their geographical distribution. These subtypes share a common a-determinant region, but differ according to mutually exclusive expression of *d* or *y*, and *w* and *r* antigenic determinants (Peterson, 1987). Why HBV has evolutionary maintained its ability to produce large quantity of sub-viral, non-infectious particles is still not clear. Possible explanations include the proposition of sub-viral particles as a decoy to divert anti-HBs antibodies away from infectious viral particles, or the direct ability of HBsAg to suppress humoral or cellular immunity.

Over the years, research into HBsAg has culminated in the development of an effective preventative vaccine. Early incarnations involved highly purified preparations isolated from the

plasma of low level carriers, which conferred high levels of protection (>92%) when tested on high-risk groups (Coutinho et al., 1983; Szmuness et al., 1980). Following this finding, a recombinant HBsAg vaccine was made and produced using yeast cell culture, and was licensed for use in humans in 1986 (McAleer et al., 1984). Recombinant HBsAg vaccines are now a standard component of routine vaccination schedules worldwide, and were recently incorporated into the vaccination schedule for the UK. Universal vaccination strategies have had significant success in reducing the prevalence of HBV and HBV-associated hepatocellular carcinoma (Chien et al., 2006); however, vaccine implementation still varies widely, reflecting the global incidence of disease (Trépo et al., 2014).

1.3 Natural history of HBV infection

Infection with HBV results in divergent clinical outcomes (Figure 1.3). Two major determinants of the outcome of HBV infection are the age and immune competence of the individual at the time of infection. In adult-acquired HBV, >95% of cases result in clearance of HBsAg, termed acute-resolving infection. Comparatively, perinatal infection in neonates and infection in young children, more commonly results in an initial subclinical phase and high rates of chronic infection. Numerically, this equates to viral persistence in 1.5% of infected adults, 20-30% of those infected as young children and up to 90% of perinatally infected individuals (Liaw and Chu, 2009).

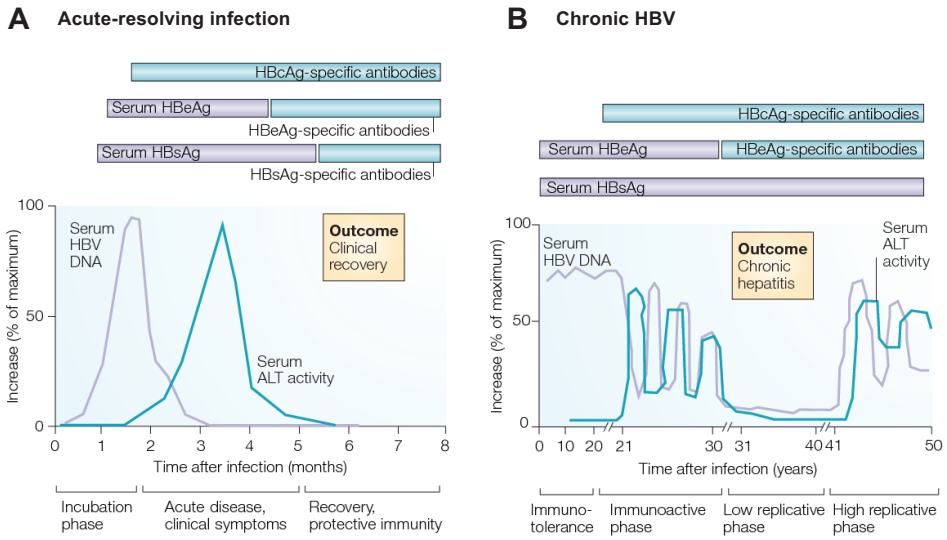

Figure 1-3 Natural history of HBV infection

A. Schematic depicting the immune response in acute infection with HBV through horizontal transmission, characterised by sequential peaks of HBV DNA and serum ALT. Clinical recovery, characterised by detection of anti-HBs and HBV-specific T cells is shown. **B.** Chronically evolving HBV resulting from vertical transmission, characterised by several phases of variable length (for details, see main section 1.3.2.1). [Clinical phases of disease correspond to those described in 3.3.2.1 as follows: 'Immunotolerant' = $HBeAg^+$ infection; 'Immunoactive phase' = $HBeAg^+$ hepatitis; 'Low replicative phase' = $HBeAg^-$ infection; 'High replicative phase' = $HBeAg^-$ hepatitis. Adapted from (Rehermann and Nascimbeni, 2005)]

1.3.1 Acute-resolving infection

HBV infection acquired in adulthood is typified by an initial quiescent phase, whereby infection can remain subclinical for a number of weeks (Figure 1.3). Following this period, HBV replication enters an exponential phase, during which levels of HBV DNA rapidly rise and peak prior to the onset of clinical symptoms and the induction of an adaptive T cell-mediated response. This symptomatic phase of disease classically corresponds with an increase in serum alanine transaminase (ALT) levels; an enzyme that is released from dying hepatocytes and thus used as a readout for liver inflammation and damage. HBV-specific antibodies associated with viral control are detected following clearance of the virus and a reduction in ALT. The presence of antibodies against HBsAg (anti-HBs), together with HBV-specific memory T-cells, provides long-term viral control and protection against reinfection and reactivation of HBV.

Due to limitations in obtaining samples from patients at the earliest pre-symptomatic stages of HBV infection, the majority of our understanding of the immune response in acute-resolving HBV has utilised models of HBV infection in chimpanzee and murine models, and the closely related woodchuck hepatitis virus infection. As HBV does not demonstrate natural tropism for murine hepatocytes, numerous models have been designed based on the introduction of human hepatocytes, including the production of chimeric mice bearing human hepatocytes and transgenic mice that express NTCP or HBV viral proteins. However, these are not without problems: for instance, chimeric mouse often display profound immunodeficiency, and transgenic mice often fail to effectively recapitulate the entire viral life cycle (Guo et al., 2018; Iannacone and Guidotti, 2015).

Nonetheless, animal models have revealed important insights into the innate and adaptive immune responses involved in HBV viral clearance. In stark contrast to most other acute viral infections, including HCV, early responses to HBV are characterised by a notable lack of innate immunity. Type I interferons are generally produced by infected cells and trigger the transcription of interferon stimulated genes (ISGs) that in turn control intracellular antiviral pathways that help to limit infectivity, for instance by reducing viral replication and spread (Wieland and Chisari, 2005). This was first demonstrated in an experimental model of HBV infection in chimpanzees; genomic analysis in HBV-infected chimpanzees identified a lack of upregulated Type I ISGs following viral infection and early expansion (Wieland et al., 2004). Building on this finding, temporal analysis of patients with acute HBV infection demonstrated delayed and impaired production of pro-inflammatory cytokines, including type I interferons, within the first 30 days of infection, in comparison to acute hepatitis A, HIV and HCV infection (Dunn et al., 2009; Stacey et al., 2009). In avoiding the induction of ISGs, HBV is said to behave like a *'stealth virus'*, whereby it can spread throughout the liver without alerting the innate immune response. How the virus achieves this is remains a subject of much debate; it is not yet understood whether the virus 'hides' from the innate response, by sequestering cccDNA or RNA/DNA intermediates to the cell nucleus or cytoplasmic

core particles, or is instead able to actively repress its induction (Durantel and Zoulim, 2009; Maini and Gehring, 2016; Tan et al., 2015; Tang et al., 2018; Wieland and Chisari, 2005). Likely, HBV evasion of innate immune responses is a combination of both limited sensing and active suppression (Maini and Gehring, 2016). Recent evidence using livers from patients with CHB demonstrated an absence of ISGs in hepatocytes infected with HBV, yet ISGs were sufficiently induced following pattern-recognition receptor (PRR) engagement using TLR-3 ligands or Sendai virus stimulation (Suslov et al., 2018). HBx protein and HBV-induced miRNA have also been implicated in the inhibition of intracellular machinery, including signalling mediated by cytosolic sensory molecules, such as retinoic acid-induced gene-I (RIG-I) (Hou et al., 2016; Sato et al., 2015; Tan et al., 2015). HBV is able to abort cell-intrinsic immunity in hepatocytes through actively suppressing TLR-3 and RIG-I pathways, resulting in weak and transient production of Type I interferons and antiviral cytokines, including IL-6 (Luangsay et al., 2015). These data suggest that an inability to activate PRR's, either through evasion or active suppression, may be one mechanism by which HBV evades the innate immune response.

Due to the poor induction of innate intracellular immunity, adaptive immune responses during acute-resolving infection are essential for viral clearance, and are efficiently and timely induced following HBV replication; however, in contrast to most other acute infections, viral clearance can take several months to establish. The exponential phase of HBV replication is accompanied by robust production of TNFα and IFNγ, that clear the majority of the viral burden whilst avoiding the induction of liver damage, as indicated by raised levels of ALT (Figure 1.3). As such, the majority of HBV clearance is deemed to be non-cytolytic and mediated by the direct antiviral effects of TNFα and IFNγ. Production of these cytokines has been associated mainly with an efficient HBV-specific T cell response, as demonstrated in transgenic mice and chimpanzees (Guidotti et al., 1996, 1999).

Accordingly, patients who spontaneously recover from HBV demonstrate robust induction of strong and multi-epitope specific CD4 and CD8 T cell responses. HBV-specific CD8 T cells accumulate in the blood and liver during HBV infection (Maini et al., 1999; Thimme et al., 2003). Depletion of CD8 T cells at the peak of viraemia delays viral clearance until CD8 T cells return, providing direct evidence for the principal role of CD8 T cells in resolution of infection (Thimme et al., 2003). However, in addition to secreting antiviral cytokines that suppress HBV gene expression and replication, HBV-specific CD8 T cells also contribute to viral clearance through the lysis of infected hepatocytes (Thimme et al., 2003) and recruitment of inflammatory cells to the liver microenvironment (Kakimi et al., 2001). Thus, despite their crucial role in viral clearance, the cytotoxic ability of CD8 T cells to kill infected hepatocytes, and their role in driving liver inflammation, are thought to be key factors underlying liver damage in HBV infection. As such, their peak abundance often coincides with the peak of liver damage (Fisicaro et al., 2009; Guidotti et al., 1999; Webster et al., 2000).

Functional CD4 T cell responses are likewise essential to the co-ordination of adaptive antiviral immune responses. CD4 T cells regulate the efficacy of CD8 T cell responses, and thus their frequency and function positively correlate with HBV clearance (Chisari and Ferrari, 1995; Guidotti et al., 2015a). Accordingly, patients with resolved HBV demonstrate robust and multi-specific CD4 T cell responses, whilst CD4 T cells in CHB characteristically have a narrow repertoire of responses and much weaker proliferative capacity (Ferrari et al., 1990, 1991). The central role of CD4 T cells in influencing the outcome of infection is supported by the *in vivo* clinical observation that HIV-infected patients with low CD4 T cell counts have an increased risk of developing chronic HBV infection following horizontal transmission (Puoti et al., 2006). Moreover, whilst depletion of CD4 T cells at the peak of viraemia does not affect HBV resolution, depletion prior to HBV infection results in qualitative and quantitative impairments in HBV-specific CD8 T cell responses, resulting in HBV persistence (Asabe et al., 2009; Thimme et al., 2003). These data point to an indirect role for CD4 T cells in early virus control, likely through the priming of CD8 T cell responses via the secretion of cytokines such as IL-2 (Zhang et al., 2009) and through the activation/licensing of professional antigen-presenting cells, which in turn activate CD8 T cells (Schoenberger et al., 1998). In addition, CD4 T cells also have a crucial role in the initiation of neutralising antibody responses.

Natural killer (NK) cells may also contribute to viral clearance (Yang et al., 2010). TNFα and IFNγ are secreted by NK cells and natural killer T (NKT) cells at sufficient levels to control HBV replication. Stimulation of NK cells in transgenic mice promoted IFNγ production by intrahepatic NK and NKT cells, resulting in the non-cytopathic inhibition of viral replication (Kimura et al., 2002). More recently, the administration of TLR-7 agonists in chimpanzee models of HBV induced both type I interferon responses and IFNγ production by activated NK cells, resulting in clearance of HBV-infected cells (Lanford et al., 2013). Importantly, NK cells are enriched in the liver compared to the blood (including during HBV infection), where they can account for up to 40% of the lymphocyte population and contribute to antiviral immunity (Doherty et al., 1999; Maini and Peppa, 2013). Although classical iNKT cells are only present at very low numbers in human livers, an analogous population of mucosal-associated invariant T (MAIT) cells are abundant and can produce large quantities of IFNγ (Garner et al., 2018; Jo et al., 2014; Loh et al., 2016; Tang et al., 2013; Ussher et al., 2014).

Although the virus is likely never eradicated completely, resolution of HBV provides an informative model for functional cure, demonstrating that viral replication can be controlled by functional T cell responses (Penna et al., 1996; Rehermann et al., 1996). How much the humoral response contributes to viral control is poorly understood at present; however, it probably has a more important role than previously thought (discussed in detail in section 1.6).

1.3.2 Failure of immune control and chronic HBV

Chronic HBV infection (CHB) is characterised by the persistence of HBsAg in the absence of anti-HBs. Mechanisms underpinning immune dysregulation in CHB have yet to be fully elucidated. However, it is clear that the failure of immune control in patients with CHB is multifactorial; viral persistence is thought to be a result of: immune exhaustion resulting from high-dose antigen stimulation ± direct effects of viral proteins; continued production of stable forms of HBV (including cccDNA); and HBV tropism for the tolerogenic environment of the liver. In persistent infection, the immune response fails to control the virus and can instead trigger tissue damage, leading to cirrhosis and ultimately liver cancer.

1.3.2.1 Clinical manifestations in CHB

Chronic infection with HBV manifests as several distinct phases of disease, reflective of the interaction between HBV replication and the host immune response. The natural course of CHB infection is divided into four clinically distinct phases based on a combination of virological, serological and biochemical parameters (Table 1.1; Figure 1.3). These have recently been re-named according to positive or negative detection of secretory HBeAg, and to whether there is simply ongoing infection or evidence of liver disease (i.e. hepatitis). The immune mechanisms that dictate these different phases and mediate the transition between them have yet to be fully established.

Table 1-1 Natural history and classification of patients with CHB based on virological, serological and biochemical parameters

Based on European Association for the Study of Liver (EASL) clinical guidelines (European Association for the Study of the Liver, 2017). Clinical phases of disease previously referred to as follows: 'Immunotolerant' = $HBeAg^+$ infection; 'Immunoactive phase' = $HBeAg^+$ hepatitis; 'Low replicative phase' = $HBeAg^-$ infection; 'High replicative phase' = $HBeAg^-$ hepatitis.

	$HBeAg^+$ Chronic infection	**$HBeAg^+$ Chronic hepatitis**	**$HBeAg^-$ Chronic infection**	**$HBeAg^-$ Chronic hepatitis**
HBsAg	High	High/intermediate	Low	Intermediate
HBeAg	Positive	Positive	Negative	Negative
HBV DNA	$>10^7$ IU/ml	10^4-10^7 IU/ml	<2000 IU/ml	≥2000 IU/ml
ALT	Normal	Elevated	Normal	Elevated
Liver disease	None/minimal	Moderate/severe	None	Moderate/severe

HBeAg+ chronic infection is characterised by the co-occurrence of extremely high levels of HBV DNA with a normal or minimally raised ALT levels, indicative of a lack of liver inflammation/damage. As a result, patients demonstrate minimal or no liver fibrosis, yet high levels of HBV DNA integration in hepatocytes suggests that early pro-carcinogenic changes may already be underway (Kennedy et al., 2017). HBeAg+ chronic infection is more commonly detected in young patients following vertical infection by HBeAg+ mothers, and can persist for 20-30 years, in contrast to those who acquire HBV infection in adulthood in whom this period is short or even absent.

The mechanisms associated with the loss of immunotolerance and the transition through to active disease phase are still mostly unknown, although previous work from our group has suggested a role of arginase-expression myeloid derived suppressor cells in maintenance of the immunotolerant/HBeAg+ chronic infection phase (Pallett et al., 2015). HBeAg+ chronic hepatitis is characterised by fluctuating levels of HBV DNA and raised ALT levels. Accordingly, HBeAg+ chronic hepatitis is associated with progression of liver fibrosis, attributable to immune-mediated responses against the virus. HBeAg+ chronic hepatitis is more frequently (and more rapidly) initiated in patients infected during adulthood.

Seroconversion against HBeAg is often accompanied by a non-replicative or inactive phase, termed HBeAg- chronic infection. During this phase, patients have undetectable or low levels of HBeAg coupled with normalised ALT values. These patients have a low risk of progression to cirrhosis or HCC, assuming that they remain within this phase. Approximately 1% of patients within this phase spontaneously clear HBsAg and/or produce detectable anti-HBs (Villa et al., 2011). This period of inactive disease can be long-lasting; however, HBV reactivation is known to occur in a subset of patients, characterised by fluctuating levels of HBV DNA and ALT (referred to as HBeAg+ chronic hepatitis). HBV reactivation is associated with recurrent necroinflammatory liver disease and fibrosis. The majority of cases of HBV reactivation can be attributed to HBV variants in the precore and core promoter regions, that lead to evasion of protective immune responses whilst limiting re-expression of HBeAg (Fattovich et al., 2008).

1.3.3 Mechanisms of lymphocyte dysfunction in CHB

Persistent infections are commonly characterised by weak and mono-specific T cell responses. Depletion of virus-specific T cell responses is particularly evident in patients with CHB, who demonstrate profoundly decreased levels of *ex vivo* HBV-specific CD8 T cells compared to those with acute infection (Boni et al., 2007; Maini et al., 2000). Accumulating evidence over recent years has described mechanisms by which HBV-specific T cells are depleted in persistent infection. Gene expression profiling of HBV-specific T cells identified high expression of the pro-apoptotic molecule, Bcl-2-interacting mediator (Bim), in cells isolated from patients with chronic infection

relative to those in which it resolved (Lopes et al., 2008). Elevated Bim expression was confirmed at the protein level and was associated with premature apoptosis of HBV-specific T cells both *ex vivo* and in culture; in opposition, blockade of Bim rescued HBV-specific T cells from premature cell death. An increased propensity towards premature cell death, also observed in intrahepatic and peripheral HCV-specific T cells (Radziewicz et al., 2008), is postulated to be driven by the nature of T cell activation within the tolerogenic liver. This concept is supported by the demonstration that antigen presentation by hepatocytes results in T cells prone to premature death through the upregulation of Bim (Bowen et al., 2004; Holz et al., 2008). In addition, intrahepatic CD8 T cells may be further depleted through interactions with liver-infiltrating and liver-resident NK cells, which upregulate the death ligand TNF-related apoptosis-inducing ligand (TRAIL), enabling them to kill HBV-specific T cells expressing high levels of the death receptor, TRAIL-R2 (Das et al., 2008; Peppa et al., 2013; Stegmann et al., 2016).

Cells that resist depletion are often profoundly dysfunctional. HBV-specific CD8 T cells in patients with high levels of viral replication and extensive liver damage show markedly decreased proliferative capacity compared to those with low level viral replication and minimal or absent liver disease (Maini et al., 2000), in line with similar data showing reduced functional capacity of CD8 T cells and deletion of immune-dominant clones in murine models of chronic LCMV infection (Wherry et al., 2003). Mechanisms underpinning dysfunction in HBV-specific T cells are outlined below and summarised in Figure 1.4.

1.3.3.1 Viral exhaustion of lymphocytes

CD8 T cells show a gradual decline in effector function following persistent antigen stimulation. This state is referred to as 'exhaustion' and contributes to impairment of antiviral responses in settings of chronic viral infections and cancer; in opposition, this reduction in function also has a role in avoiding extensive tissue damage in the context of antigen persistence.

Exhaustion within T cells typically manifests as hierarchical loss of effector function, altered expression of transcription factors and checkpoint molecules, and metabolic dysregulation (Wherry et al., 2003). T cell exhaustion can also impair memory responses: exhausted virus-specific T cells display a loss of maintenance of memory responsiveness independent of antigen, indicative of dependence on cognate antigen for long-term persistence (Shin et al., 2007a). Exhausted virus-specific T cells were first described in models of chronic LCMV, with functional exhaustion ascribed to loss of cytokine production, namely IFNγ (Zajac et al., 1998). Analogous defective T cells have since been identified in human infections (reviewed in Wherry and Kurachi, 2015), including chronic HBV (Boni et al., 2007), where the combination of persistently high viral load and tolerogenic environment of the liver are thought to drive exhaustion. In CHB, persisting HBV-

specific T cells produce less IFNγ, have a reduced proliferative capacity and exhibit a phenotype associated with exhaustion.

Central to T cell exhaustion are pathways involved in immune regulation. Activation of lymphocytes is carefully regulated, requiring a delicate balance between activation of effector cells in response to pathogenic challenge whilst maintaining peripheral tolerance against *'self'* antigen. This is accomplished through immune checkpoints that act as brakes for the cell and repress its function: cells must overcome these co-inhibitory signals in order to perform their full effector function. Persistent TCR stimulation in the context of chronic infection leads to the upregulation of a number of co-inhibitory molecules, including cytotoxic T-lymphocyte associated protein 4 (CTLA-4), T cell immunoglobulin gene and mucin-3 (Tim-3) and programmed cell death protein (PD-1), partially attributable to the upregulation of nuclear factor of activated T cells (NFAT) (Martinez et al., 2015). Although viral persistence is required for the induction of lymphocyte exhaustion, T cell functionality in CHB does not clearly correlate with quantities of viral protein detectable in patient serum (Loggi et al., 2013), with varying levels of T cell restoration achieved following NUC-treatment and HBsAg clearance (Boni et al., 2012). Importantly, expression of co-inhibitory molecules results in suppression of effector function in instances where excess co-inhibitory signals outweigh co-stimulatory signals (Blackburn et al., 2009).

The best characterised immune checkpoint within the context of chronic viral infection is PD-1. Upon binding to its ligands PD-L1/L2, PD-1 associates with the TCR, in combination with the phosphatase, SHP-2. Together, these induce the de-phosphorylation of proximal TCR signalling molecules, resulting in the suppression of TCR signalling (Yokosuka et al., 2012). However, more recent work has shown that PD-1 also has a more potent role in de-phosphorylation and suppression of the T cell co-receptor CD28 (Hui et al., 2017), indicating additional mechanisms by which PD-1 restrains T cell activation. In addition, PD-1 ligation has been suggested to upregulate expression of the transcription factor BATF, which in turn impairs proliferation and cytokine production by T cells (Quigley et al., 2010). These data suggest that development of T cell exhaustion is an active process that can be recovered through blockade of co-inhibitory molecules.

Numerous studies have now identified elevated expression of co-inhibitory molecules on virus-specific T cells in HBV associated with impaired effector function (Boni et al., 2007; Fisicaro et al., 2012; Nebbia et al., 2012; Schurich et al., 2011). PD-1 shows the highest level of expression relative to other co-inhibitory molecules, identifiable on approximately 80% of HBV-tetramer staining T cells and rising to almost 100% of HBV-specific T cells in the liver (Bengsch et al., 2014). This is exacerbated by the high levels of PD-1 ligands expressed by hepatocytes and non-parenchymal cells in the liver, that increase further in the context of viral infection and impair local effector responses (Protzer et al., 2012).

To this end, blockade of PD-1 has shown promising results in re-invigorating host immune responses in cancer treatment. Blockade of PD-L1 resulted in increased frequencies of HBV-specific T cells *in vitro*, but showed significant heterogeneity in responses between individuals tested (Bengsch et al., 2014). PD-1 blockade also increased the frequency of IFNγ and IL-2 producing cells, showing much greater rescue of intrahepatic T cell function, compared to cells isolated from the periphery (Fisicaro et al., 2010). The addition of IL-12 in combination with PD-L1 blockade significantly enhanced IFNγ production by HBV-specific T cells, as did blockade of another checkpoint molecule, CTLA-4 (Schurich et al., 2011, 2013).

Although lymphocyte exhaustion is most commonly discussed in relation to CD8 T cells, growing evidence now suggests that additional immune cell populations also display evidence of 'exhaustion'. Like CD8 T cells, CD4 T cells also show signs of impaired proliferation and weak cytokine production in models of chronic infection, including LCMV (Antoine et al., 2012) and chronic HCV (Ulsenheimer et al., 2003). Blockade of PD-1 ligands resulted in release of HCV-specific T cell proliferation and cytokine production (Raziorrouh et al., 2011). In comparison, illustration of exhausted CD4 T cell responses in CHB is limited. HBV-specific CD4 T cells demonstrate upregulated expression of inhibitory molecules including PD-1 and TIM-3 (Nebbia et al., 2012; Raziorrouh et al., 2014), in line with a supposed exhausted phenotype (Ye et al., 2015). However, as yet, the functional consequences of CD4 T cell exhaustion in CHB remain poorly understood.

1.3.3.2 Altered metabolism of HBV-specific T cells

It is increasingly recognised that defects in cellular metabolism contribute to the profound functional exhaustion of HBV-specific CD8 T cells. Dynamic metabolic reprogramming is essential for lymphocyte effector function and memory formation; upon activation, lymphocytes must undergo metabolic reprogramming in order to meet increased demand. Exhausted PD-1^{hi} HBV-specific T cells demonstrate upregulated expression of the glucose transporter, Glut-1, in line with their dependence on glucose to maintain effector cytokine expression. Upon glucose withdrawal, HBV-specific T cells were unable to utilise oxidative phosphorylation in contrast to more functional CMV-specific T cells from within the same patient, likely due to underlying defects in mitochondria (Schurich et al., 2016). This was supported by data demonstrating a link between impaired mitochondrial polarisation and function with virus-specific T cell exhaustion; accordingly, improvement of mitochondria via targeted antioxidant treatment resulted in the functional restoration of HBV-specific T cell responses (Fisicaro et al., 2017). Metabolic pathways, including both oxidative phosphorylation and glycolysis, appear to be modulated early in chronic infection, which appears to be in part mediated by expression of PD-1 (Bengsch et al., 2016).

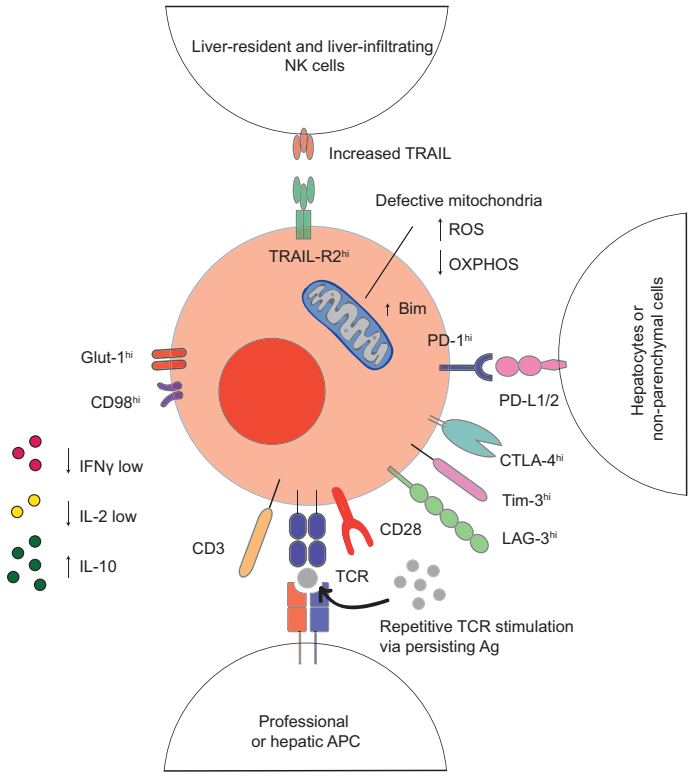

Figure 1-4 Schematic outlining the features of T cell defects in the context of chronic HBV infection

Diagram depicting mechanisms associated with T cell dysfunction in CHB, including: i. defective metabolism and mitochondrial polarisation; ii. repetitive TCR signalling and upregulation of inhibitory receptor expression; iii. elevated expression of the pro-apoptotic mediator Bim. [Adapted from (Maini and Pallett, 2018). Abbreviations used: Glut-1 = glucose transporter 1. Trail = tumour necrosis factor-related apoptosis-inducing ligand. TCR='T-cell receptor. ROS=reactive oxygen species. MHCI=MHC class I]

1.3.4 A role for failed humoral immunity in viral persistence?

An additional mechanism that may contribute to failure of immune control in CHB and that has been understudied to date, is a defect in the frequency or function of antiviral B cells. B cells are responsible for the production of neutralising antibodies that can limit infectivity and promote clearance of the virus. Typically, non-cytopathic viruses such as HBV induce delayed low titre neutralising antibody responses due to decreased pressure pertaining to the survival of the host and a strong reliance on T cell toxicity for viral clearance. However, it is also possible that HBV is able to deplete/modify B cell subsets, subvert CD4 T cell responses leading to abnormal B cell activation, and/or induce pathological changes in secondary lymphoid organs: all factors that may contribute to impaired neutralising responses during non-cytopathic viral infection (Hangartner et al., 2006). To understand how B cell responses may contribute to HBV persistence, it is first important to consider the role of B cells in antiviral responses.

1.4 B cell responses in viral infections

The ability of the adaptive immune response to respond to a wide range of pathogens is beholden in the diversity of the antigen receptor repertoire for T and B cells. B cell antigen-specificity is proffered by the expression and production of immunoglobulins. Membrane-bound immunoglobulin serves as the receptor for antigen, referred to as the B cell receptor (BCR). As the constant region of the immunoglobulin is inserted into the cell membrane, the antibody itself does not exert an effector function; instead, the exposed variable regions of the immunoglobulin are responsible for recognising and binding antigen, leading to B cell activation, clonal expansion and specific antibody production. Antibodies are produced by terminally differentiated B cells and span a vast range of specificities - each B cell clone producing one specific immunoglobulin. Antibody production by B cells of a single-antigen specificity was demonstrated in a seminal paper by Nossal and Lederberg, who showed that single-cell suspensions of B cells isolated from mice inoculated with two different types of *Salmonella* antigen produced an immobilising agent – presumed to be antibody – to only one of the antigens (Nossal and Lederberg, 1958).

The development of humoral responses requires virus-specific B cells to encounter viral antigens in secondary lymphoid organs, become activated, proliferate and enter specific differentiation programmes. Viruses are able to induce B cell activation as a function of their combined ability to cross-link the BCR and to deliver RNA/DNA signals to toll-like receptors. The efficacy of viruses to activate B cells is dependent on various factors, including the size of the viral particle, its geometry and its relative abundance (Zabel et al., 2013). B cell expression of cell-surface molecules can also modulate activation; for example, engagement of the B cell co-receptor molecule CD21 by complement-coated viral particles, significantly reduces the threshold of stimulation required for B cell activation and IgG production (Jegerlehner et al., 2002). Distinct from their production of antibodies, B cells can also exert additional antiviral functions, such as their ability to secrete cytokines (discussed in more detail in section 1.4.7).

1.4.1 Early B cell development

Initial diversity of the BCR is imparted through a complex rearrangement of germline immunoglobulin (*Ig*) genes in the bone marrow that occurs throughout early B cell development. B cells are generated from lymphoid precursors in the bone marrow, whereby cells progress through pro-B cell and pre-B cell stages of development. Stages of B cell development in the bone marrow are defined by phases of *Ig* gene arrangement of the pre-BCR, whereby V, D and J segments encoding the variable region of the *Ig* heavy and light chains recombine to form a functional BCR. This mechanism creates combinatorial diversity through the random pairing of V D J gene segments (within the heavy chain) or V J gene segments (within the light chain) resulting in large number of possible unique configurations. Additional diversity during V (D) J

recombination arises by the introduction or deletion of nucleotides at the junction of segments as they are linked together (Schroeder and Cavacini, 2010). Recombined heavy and light chains are then randomly paired to form an intact antibody molecule and tested for auto-reactivity; B cells that do not react strongly to self-antigen leave the bone marrow as immature transitional B cells, before eventually maturing into IgM^+IgD^+ mature naïve B cells. At rest, homeostasis of peripheral mature B cells is regulated by the co-ordination of several receptors, including the BCR and B cell activating factor receptor (BAFF-R). Deletion of the BCR - or associated transmembrane proteins CD79a and CD79b - results in the death of mature B cell subsets (Kraus et al., 2004; Lam et al., 1997), whist blockade of BAFF-R, or its ligand BAFF/BlyS, leads to the loss of follicular and marginal zone B cells in mice (Gross et al., 2001; Rauch et al., 2009). Studies have since revealed a critical interplay between these two pathways, whereby BAFF-R signalling results in the phosphorylation of CD79a and spleen tyrosine kinase (Syk); inducible deletion of *Syk* in mature B cells resulted in a loss of ~80% of follicular B cells (Schweighoffer et al., 2013). These data suggest that BAFF-R derived survival signals are transduced via the BCR and activation of Syk.

1.4.2 Progression of B cell differentiation during infection

1.4.2.1 Antigen recognition within secondary lymphoid organs

For the most part, B cell activation and clonal expansion following antigen-activation occurs within secondary lymphoid organs, the best characterised of these being the lymph node. Viruses that evade first-line innate immune responses are disseminated systemically via the lymphatic system, whereupon they encounter B cells in draining lymph nodes. These provide a specialised micro-environment for the presentation of virus-derived antigens and the activation of naïve B and T cell responses (von Andrian and Mempel, 2003; Denton and Linterman, 2017; Gatto and Brink, 2010). Similar encounters are also detectable within the spleen, which filters blood-borne viruses enabling antigen capture and presentation by specialised populations of macrophages and dendritic cells. As such, the spatiotemporal dynamics of B cell activation in lymph nodes are closely mirrored by those occurring in the spleen (Heesters et al., 2016).

The structure of a lymph node is broadly characterised by follicles comprised of IgM^+IgD^+ naïve B cells (referred to as B cell follicles), bordered by T cell rich areas (referred to as the T cell zone) (Figure 1.5). Antigen is shuttled into the lymph node via monocytes, dendritic cells, macrophages and marginal zone B cells, whereupon it is deposited on follicular dendritic cells (FDCs) and presented to B cells within the germinal centre. Engagement of BCRs with antigen retained on FDCs is critical for GC B cell activation, GC development, somatic hypermutation and affinity maturation, and the induction of secondary antibody responses (El Shikh and Pitzalis, 2012).

Early antigen interactions can also take place in the sup-capsular sinus (SCS) of the lymph node, defined as the space between the cortex (consisting of the B cell follicle and T cell zone) and the outer capsule (Kuka and Iannacone, 2014) (Figure 1.5). The SCS receives unfiltered lymph from afferent lymph vessels, and is populated with SCS macrophages that help to translocate large antigens, including immune complexes and inactivated viruses, to follicular B cells (Carrasco and Batista, 2007; Phan et al., 2007). Although small soluble antigens, less than ~70 kD in size, can readily infiltrate the B cell follicle (Pape et al., 2007), SCS macrophage capture of larger particulate antigens (e.g. intact viral particles) represents an important mechanism in the induction of antiviral humoral responses.

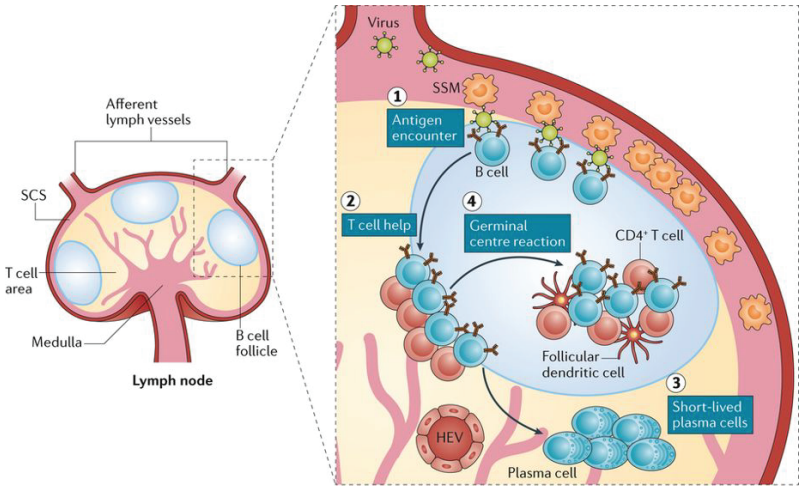

Figure 1-5 Schematic of the structure of the lymph node

Schematic of the lymph node, showing the sub-capsular sinus (SCS), T cell zone (area) and B cell follicle. Viruses can enter the lymph node via the afferent lymph where they are captured and retained by SCS macrophages (SSMs). Antigen-activated B cells relocalise to the B – T cell border in order to receive T cell help, and either differentiate into extra-follicular plasmablasts or localise back to the follicle and enter the germinal centre. [Figure adapted from (Kuka and Iannacone, 2017)]

Naïve B cells are recruited to the B cell follicle via expression of CXCR5, allowing them to scan CXCL13-expressing follicular dendritic cells for cognate antigen (Ansel et al., 2000; Suzuki et al., 2009). Upon antigen-recognition and binding, B cells undergo rapid proliferation and migrate towards the T - B border via the upregulation of the chemokine receptor CCR7, responding to gradients of CCL19 and CCL21 (Okada et al., 2005; Reif et al., 2002). Here, they form stable interactions with CD4 T cells that home to the B cell follicle via upregulation of CXCR5, whereupon they differentiate into T follicular helper cells (T_{FH}) (Ansel et al., 1999). These crucial interactions facilitate the activation and proliferation of both B and T cells in a bidirectional manner.

1.4.2.2 T cell help

Following antigen-activation, the BCR delivers bound antigen to intracellular sites where it is degraded and presented at the cell surface bound to MHC Class II molecules. Peptide - MHC molecules are then available to antigen-specific T helpers, activated by the same pathogen, which deliver activating signals to the B cell (known as *linked recognition*). The co-operation between CD4 T cells and B cells was first demonstrated by experiments showing that adoptive transfer of either B cells or T cells alone into irradiated mice was insufficient to generate a robust antibody response (Mitchell and Miller, 1968, 1968). Since then, many mechanisms have been identified that promote B cell activation (Crotty, 2015), including T cell production of IL-4, IL-21 and CXCL13, and interactions between CD40 and CD40-L, expressed on B cells and CD4 T cells respectively. Ligation of CD40 on naïve B cells, in particular, is key to B cell activation, and is required for their clonal expansion and proliferation (Armitage et al., 1993; Noelle et al., 1992; Nonoyama et al., 1993).

Within the B cell follicle, T_{FH}-driven expression of IL-21 is postulated to stimulate differentiation to plasma cells, and along with IL-4, can induce class-switch recombination and somatic hypermutation in B cells. In this way, cytokine signals from T helper cells play a key role in driving the molecular events preceding class-switch recombination in B cells, whereby antigen-activated mature naïve B cells downregulate expression of IgM and IgD, and instead switch to expressing IgG, IgA or IgE with distinct effector functions. Finally, CXCL13 expression by T_{FH} recruits $CXCR5^+$ B cells to co-localise and form functional associations with T_{FH}, whilst T_{FH} expression of SAP helps to sustain T-B cell interactions, allowing sufficient time for B cell help to occur (Qi et al., 2008).

1.4.2.3 Extra-follicular responses

Before differentiation into effector or memory B cell subsets, antigen-activated B cells have an initial fate decision: to enter the germinal centre (becoming a 'germinal centre B cell'; GC B cell), differentiate into a germinal centre-independent memory B cell, or leave the B cell follicle as a short-lived plasma cell (or 'extra-follicular plasma cell'). This decision is thought to be in part mediated by the antigen reactivity of the BCR, whereby high-affinity B cells are more likely to generate short-lived responses. Using a library of hen egg lysozyme (HEL) mutant proteins with divergent affinities for the BCR, Paus et al., showed that decreasing the affinity led to impaired formation of extra-follicular plasma cell responses but did not affect germinal centre responses (Paus et al., 2006). In contrast, low affinity interactions instead predisposed B cells towards long-lived plasma cells and memory B cells (O'Connor et al., 2006).

In T cell-dependent immune responses to viruses, the early antibody response is provided by activated B cells that differentiate into extra-follicular plasmablasts, transitioning to long-lived

plasma cells later in the humoral response. Extra-follicular plasmablasts are short-lived and generate much of the early-induced antiviral antibody responses; accordingly, antibody-secreting cells (ASCs) can be detected as early as three days after infection and secrete low-affinity antibody (Baumgarth, 2013; Smith et al., 1996). During active immune responses, short-lived plasmablasts migrate towards sites of inflammation through the expression of CXCR3 (Hauser et al., 2002; Muehlinghaus et al., 2005).

1.4.3 Generation of B cell memory

Protective immunity against *de novo* or secondary infection depends on the longevity and rapid responsiveness of memory responses upon re-encountering antigen. During primary immune responses, a portion of B cells activated during initial cognate interaction with T cells do not differentiate into effector cells and instead establish a population of memory B cells. Upon secondary infection and subsequent antigen challenge, these cells can re-enter follicles and develop into antibody-secreting plasma cells or re-seed the memory B cell pool (Zhang et al., 2016). During this process, additional diversity is incorporated in order to tailor the response towards the infecting pathogen. Within humoral responses, first-line defence is provided by high-affinity pre-existing antibodies secreted by long-lived plasma cells, assuming that the concentration of antibodies at the site of reinfection is sufficiently high. Where levels of antibodies are not protective, pathogen-experienced memory B cells are rapidly reactivated to produce antibodies. These secondary responses are typically faster and generate higher affinity antibodies of switched isotypes, reflective of B cells that have undergone expansion within the germinal centre.

1.4.3.1 The germinal centre

Key to the generation and diversification of protective humoral responses is the clonal expansion and maturation of antigen-activated B cells in the *germinal centre*. These are transient structures that form within peripheral lymphoid organs in response to T cell-dependent antigen, and facilitate BCR diversification and selection of high-affinity antibodies. Germinal centres were first described in 1884 as distinct micro-anatomical regions thought to persist within lymphoid organs and provide a key source of lymphocyte generation. Follow-up work demonstrated that these sites developed only in response to antigen and consist predominantly of proliferating B cells (Nieuwenhuis and Opstelten, 1984). Indeed, rapidly proliferating B cells can be identified in the spleen and lymph nodes approximately six days after immunisation (Amitai et al., 2017; Jacob et al., 1991), with germinal-centre derived memory B cells and plasma cells detectable after one week (Blink et al., 2005).

1.4.3.2 Diversification by somatic hypermutation and affinity maturation

Antigen-activated B cells are committed to differentiate into germinal centre precursors outside of the follicle; migration into the centre of follicle, where they form early germinal centre responses, is dependent on the master transcriptional regulator of germinal centre B cells, Bcl-6 (Baumjohann et al., 2011; Kitano et al., 2011).

In order to diversify their antibody repertoire and generate high-affinity responses, GC B cells undergo a process of somatic hypermutation and affinity-based selection, a process closely guided by an extensive network of stromal cells (reviewed in Denton and Linterman, 2017). Following activation, T and B cells move into the germinal centre, whereby GC B cells begin to rapidly divide and segregate into two zones, referred to as the dark and light zone (Figure 1.6). Cells populating these two zones can be distinguished by their rate of cell division. GC B cells in the dark zone are rapidly dividing, allowing them to undergo a process of somatic hypermutation that introduces random nucleotide exchanges into *Ig* genes encoding the variable region (*IgV*) (Mesin et al., 2016; Wagner and Neuberger, 1996). This process is dependent on DNA breaks, which in turn relies on the expression and activity of the enzyme activation-induced cytidine deaminase (AID). Signals delivered via T_{FH}-secreted cytokines activates AID, which initiates breakage through the deamination of cytidines directly on DNA and thus facilitates gene recombination (Gatto and Brink, 2010). Error-prone repair then leads to the introduction of somatic mutations that further diversify *IgV* regions and lead to changes in BCR specificity and affinity (Di Noia and Neuberger, 2007). The affinity of the BCR is then tested through cognate interactions with follicular dendritic cells and T_{FH} situated within the light zone of the germinal centre (Victora and Nussenzweig, 2012).

GC B cells within the light zone are non-dividing; here, cells undergo class-switch recombination and begin to differentiate into either long-lived plasma cells or memory B cells (Klein and Dalla-Favera, 2008). Newly formed GC B cell clones compete for antigen and survival signals presented by FDCs and T_{FH} cells (van Nierop and de Groot, 2002; Victora et al., 2010). Mutations that lead to increased affinity are selected for and selectively expand, whilst those that demonstrate impaired antigen binding are deleted by apoptosis (Hamel et al., 2012). As such, rates of cell division and hypermutation are directly proportional to the amount of antigen captured and presented by GC B cells to T_{FH} (Gitlin et al., 2014). Importantly, GC B cells repeatedly migrate between the dark and light zones as they undergo iterative cycles of somatic hypermutation and selection. As a result, this process results in the progressive increase in the affinity of antibodies over time. Elegant multiphoton microscopy studies in mice visualised the process by which germinal centres become homogenous, demonstrating parallel maturation of individual clones within the germinal centre. This study further proposed that spontaneous 'bursts' of individual clones leads to drastic expansion of clonal variants and facilitates higher rates of diversification resulting in improved

affinity (Tas et al., 2016). The evolution of high-affinity antibodies throughout the course of infection was demonstrated by Liao et al., who tracked the evolution of a broadly neutralising antibody response in a single HIV-infected patient from shortly after inoculation to more than three years post-infection, at which point broadly neutralising antibodies were detected (Liao et al., 2013). In sequencing antigen-specific B cells throughout the time course of infection, in combination with single-genome sequencing of HIV, they demonstrated concomitant antibody maturation and virus evolution (Liao et al., 2013). Whilst these data evidence the capacity of immune responses to evolve and mature over time, they simultaneously highlight the ability of viruses to mutate and escape adaptive immune responses.

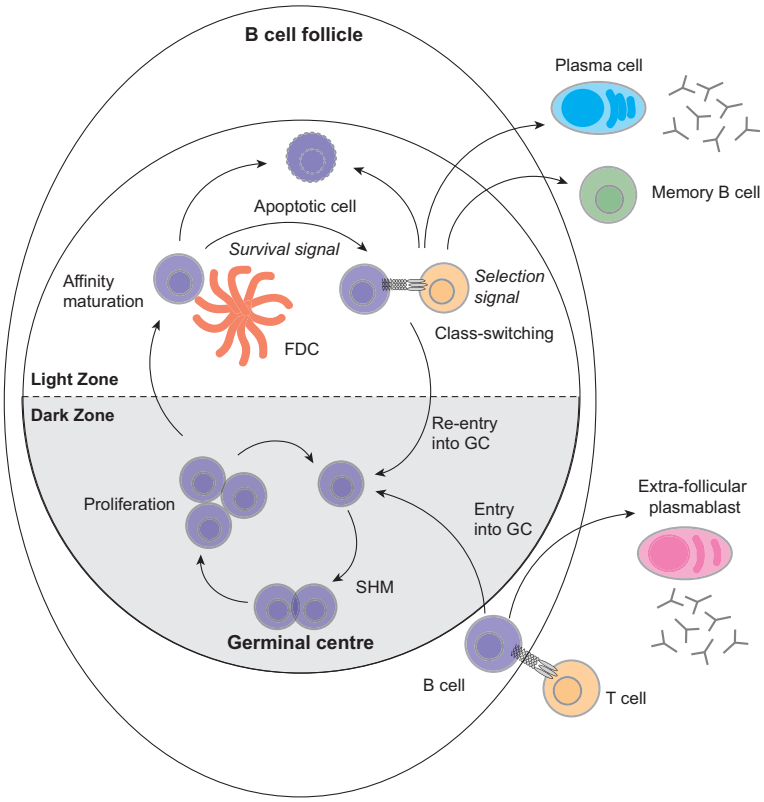

Figure 1-6 Schematic of the B cell follicle and germinal centre

Simplified schematic outlining affinity maturation in the germinal centre in the B cell follicle. B cells present antigen to T helper cells at the T cell – B cell border and receive co-stimulatory signals. Antigen-activated B cells can differentiate into extra-follicular plasmablasts or enter the dark zone, where they undergo SHM and proliferate prior to migrating into the light zone. In the light zone, mutated BCRs are exposed to antigens, expressed by follicular dendritic cells. BCRs that detect antigen with low affinity do not receive survival signals and therefore undergo apoptosis. Surviving B cells compete for T cell help, whereby the highest affinity antibodies are selected. Selected cells either re-enter the dark zone (where they undergo further rounds of SHM and proliferation), or exit the germinal centre as plasma cells or memory B cells. [Abbreviations used: GC = germinal centre; SHM = somatic hypermutation; FDCs = follicular dendritic cells. Adapted from (Heesters et al., 2014)]

1.4.3.3 Outcome of the germinal centre: memory B cells versus long-lived plasma cells

To date, no mechanism explaining the fate of germinal centre B cells (to develop into either memory B cells or plasma cells) has been identified. The current prevailing theory is that stochastic differentiation of germinal centre B cells to a memory cell fate confers a survival advantage, leading to the generation of memory B cell populations. In support of this, over-expression of pro-apoptotic factors, inducing Bcl-2 interacting mediator of cell death (BIM) and p53-upregulated modulator of apoptosis (PUMA), expands the population of $IgG1^+$ memory B cells (Clybouw et al., 2011; Fischer et al., 2007).

1.4.4 Memory B cell subsets

Following primary immune challenge and the formation of the germinal centre, memory B cells circulate throughout the body as resting B cells until reactivation upon secondary antigen encounter. Whilst continuous production of high-affinity antibody by long-lived plasma cells provides ongoing immune defence, memory B cells require re-stimulation to provide enhanced and improved immune responses, typically dependent on signals from both antigen and T_{FH} cells (Ochsenbein et al., 2000). Memory B cells can survive for decades, and differentiate far more efficiently upon antigen-specific or polyclonal stimulation than naïve B cells (Seifert and Küppers, 2016). Subsequent responses are of higher affinity than naïve B cells as a result of affinity maturation in the germinal centre. B cells that have undergone class-switching in the germinal centre lose surface expression of IgM and IgD, and instead upregulate switched immunoglobulins, including IgG, IgA and IgE. IgA^+ memory B cells are generally considered to be associated with mucosal responses, due to the role of IgA in mucosal defence (Mantis et al., 2011) and their preferential accumulation in mucosa-associated lymphatic tissue (Neutra et al., 1996). IgE^+ memory B cells are extremely rare in the peripheral blood of healthy humans, but likely play a role in asthma and allergic diseases (He et al., 2015).

Memory B cells are conventionally distinguished from naïve B cells by expression of CD27 and co-stimulatory molecules CD80 and CD86 (Maurer et al., 1990; Tangye et al., 1998).
CD27 expression is associated with production of immunoglobulin, with CD70-ligation shown to promote differentiation of memory B cells to antibody-producing plasma cells (Agematsu et al., 1997). Following T cell-derived stimulation, $CD27^+$ memory B cells proliferate and enter the cell cycle more rapidly than naïve B cells, resulting in their accelerated production of antibodies following secondary antigenic challenge (Tangye et al., 2003). Accordingly, B cells carrying somatically hypermutated genes are often characterised by expression of CD27 (Klein et al., 1998; Tangye et al., 1998).

Classically, studies addressing B cell memory have focused on IgG^+ memory B cells as the dominant antibody associated with protective responses. Dependent on the type of antigenic

challenge, IgG^+ memory B cells can express one of four different subclasses of antibody – IgG1-4 – that each proffer specific effector functions. IgG^+ memory B cells are programmed to differentiate into plasma cells following antigen engagement through the repression of the transcription factor, Bach 2 (which otherwise inhibits Blimp-1, thus suppressing plasma cell formation) and enhanced BCR signalling capacity (Kometani et al., 2013; Lutz et al., 2015). Upon secondary activation, class-switched memory B cells can undergo further remodelling to boost immune responses (McHeyzer-Williams et al., 2015).

However, additional subsets of germinal-centre independent memory B cells and IgM^+ memory B cells have been identified that may have important roles in humoral immune responses (Capolunghi et al., 2013). The identification of germinal-centre independent memory B cells in humans is at present unclear; however, IgM^+ memory B cells can represent up to 50% of peripheral blood memory cells. Although previously thought to represent an early, low-affinity subset that are redundant to IgG^+ memory, emerging evidence now points to key effector functions of these cells in secondary immune responses. These cells can be identified within germinal centres and exhibit markers of somatic hypermutation and class-switch recombination (Dogan et al., 2009). IgM^+ memory B cells appear to be more long-lived and outnumber class-switched memory B cells in the secondary response (Pape et al., 2011). In humans, these differentiate rapidly into plasma cells and show preferential homing to the B cell follicle, where they can re-enter the germinal centre response and undergo further affinity maturation and class-switch recombination (Seifert and Küppers, 2009; Seifert et al., 2015). In this way, antigen-specific IgM^+ memory B cells were shown to be highly plastic and dominate the secondary response to *Plasmodium* rechallenge by quickly reactivating to produce high-affinity antibody (Krishnamurty et al., 2016). Subsequently, IgM^+ memory B cells are postulated to provide long-lasting immunity against reinfection once IgG-mediated immunity wanes. Due to their highly plastic nature, these cells may have the potential to shape secondary immune responses in accordance with changes to the infecting pathogen. An additional population that co-expresses both IgM and IgD has also been described (Seifert and Küppers, 2016), although its contribution to humoral responses is not well understood.

Finally, a population of memory B cells displaying low to negative expression of CD21 (component of the BCR co-receptor) and CD27 (classical memory marker for B cells) has been identified in epithelial tissues and in the periphery of patients with chronic infection, autoimmunity or in aged healthy individuals (Karnell et al., 2017; Portugal et al., 2017). These cells are mostly class-switched and expressed mutated hypervariable regions (Ehrhardt et al., 2005). Importantly, these cells are thought to represent an exhausted B cell subset that is hypo-responsive to BCR stimulation and poorly secretes antibody. $CD27^-CD21^{low/-}$ B cells will be discussed in more detail in Chapter Four.

The main characteristics of different memory B cell subsets identified in humans are summarised in Table 1.2.

Table 1-2 Summary of memory B cell subsets identified in humans

Summary of the key features of memory B cell subsets in humans and the markers by which they can be identified

Subset	Identification	Role
IgG$^+$ memory	IgG$^+$CD27$^+$ CD24$^+$CD38^{int}	Produce high-affinity antibody with specialised effector function
IgM$^+$ memory	IgM$^+$IgD$^-$ CD27$^+$CD24$^+$ CD38^{int}	First-line protection Outnumber IgG$^+$ memory B cells in the secondary response Can re-enter the germinal centre and produce high-affinity antibody Demonstrate rapid and plastic production of antibodies upon re-challenge
IgM$^+$IgD$^+$	IgM$^+$IgD$^+$ CD27$^+$CD24$^+$ CD38^{int}	Naïve-like subset of memory B cells Exhibit lower levels of somatic hypermutation that IgM$^+$ and IgG$^+$ memory B cells Long-lived
IgE$^+$ memory	IgE$^+$	Involved in allergic responses
IgA$^+$ memory	IgA$^+$CD27$^+$	Associated with mucosal responses
CD27$^-$ CD21$^{low/-}$	CD19^{hi}CD27$^-$ CD21$^{low/-}$(IgG$^+$) (FcRL4/5$^+$)	Arise due to persistent stimulation in the context of autoimmunity, ageing and chronic infection Represent an "exhausted" or "dysfunctional" subset Contain mutated hypervariable regions

1.4.5 Long-lived plasma cells

A proportion of GC B cells migrate to specialised niches within the bone marrow, whereupon they differentiate into long-lived plasma cells and continue to produce vast quantities of high-affinity antibody. Long-lived plasma cells are attracted to the bone marrow niche through the expression of CXCL12 and its ligand CXCR4 (Dörner and Radbruch, 2007; Nutt et al., 2015). Several cell-extrinsic factors have been proposed that mediate survival within the bone marrow, including expression of the B cell survival factors, APRIL and BAFF, and cytokines IL-6 and TNF (Wilmore and Allman, 2017). However, plasma cells also possess several cell-autonomous features that enhance their survival and facilitate their ability to continuously produce antibody, including activation of the unfolded protein response and resistance to cell death pathways initiated by DNA damage (Benhamron et al., 2014; Calfon et al., 2002; Reimold et al., 2001; Wilmore and Allman,

2017). Accumulating data suggests that antigen-specific plasma cells can be extremely long-lived, persisting for up to 10 years following vaccination, and are maintained independently of memory responses (DiLillo et al., 2008; Hammarlund et al., 2017). Moreover, additional subsets of plasma cells have been identified that can regulate immune responses independently of antibody production. Most recently, a subset of 'natural regulatory' plasma cells has been demonstrated that secrete immune-modulatory IL-10 and have the capacity to suppress T cell responses through the expression of inhibitory receptor ligands, including LAG3, PD-L1 and PD-L2 (Lino et al., 2018).

1.4.6 Antibody effector function in antiviral responses

Antibodies recognise and bind to antigen through a specific amino acid sequence, referred to as a *complementarity determining region*, present in the variable Fab region of the antibody. However, antibody effector function is conferred by the Fc portion, which can in turn be modified by isotype switching and post-transcriptional modification. A fraction of antibodies produced by virally-activated B cells have direct antiviral activity and are referred to as neutralising antibodies (nAb). These antibodies bind directly to the viral particle and prevent entry of the virus to target cells, either through steric obstruction or through directly binding to the receptor-binding site (Corti and Lanzavecchia, 2013; Hangartner et al., 2006). The ability of these antibodies to neutralise viral particles is dependent on the avidity of the antibody for exposed antigens on the virus (Bachmann et al., 1997). nAbs are critical for long-term viral control and protection against re-infection. Accordingly, high levels of protection, obtainable by the most efficient vaccines, is closely associated with the induction of cross-reactive neutralising antibody responses (Burton and Hangartner, 2016; McCoy and Burton, 2017) (discussed in more detail in section 1.5).

The vast majority of antibodies produced during viral infection do not have neutralising capacity, predominantly because they are directed against fragments of the virus that are released from dying infected cells and therefore do not target intact antigens expressed by infected cells. However, these antibodies can still have important roles in the co-ordination of the immune response. In a similar way to neutralisation, antibodies (most commonly polymeric IgA and IgM) can also bind directly to pathogens and cause them to aggregate, thus blocking infectivity (Forthal, 2014). Antibodies that bind to the surface of viruses opsonise the pathogen and promote internalisation via Fc receptor engagement on phagocytic cells in a process referred to as antibody-dependent phagocytosis. (Excler et al., 2014; Kramski et al., 2013). Fc-receptor engagement by effector cells is likewise central to facilitating antibody-dependent cell-mediated cytotoxicity (ADCC) - an effective pathway in the clearance of infected cells. This occurs when an antibody cross-links antigen expressed by the target cell and Fc-receptors on effector cells, initiating a cascade of events that results in the death of the pathogen or the pathogen-infected cell (Forthal and Moog, 2009; Jennewein and Alter, 2017). Such Fc-mediated effector functions of antibodies are associated with favourable outcomes

in numerous contexts of chronic infection, including HIV (reviewed in Lewis, 2014). However, how Fc-mediated protection is affected in chronic infections is less well understood. In chronic HCV, activated NK cells that downregulate expression of the Fc-receptor CD16, result in impaired ADCC-mediated killing of infected target cells and may represent a previously unappreciated mechanism underpinning viral persistence (Oliviero et al., 2017).

Antibodies also activate the complement cascade resulting in the generation of complement-derived products that target the cell for degradation (Karsten and Köhl, 2012). This effect is predominantly mediated by the aggregation of IgM and IgG3 antibodies to the surface of the infected cell or pathogen, that recruit C1q and initiate a cascade resulting in the formation of the membrane attack complex and lysis of the pathogen or infected cell (Diebolder et al., 2014; Jennewein and Alter, 2017; Noris and Remuzzi, 2013). The extent by which complement is able to induce cell lysis is considered to be dependent on the level of antigen expression on the cell surface (Olivier Schwartz, unpublished data). This role of complement in enhancing antibody responses is demonstrated in individuals that have lost critical components of the complement cascade and have impaired primary and secondary responses to antigen (Sörman et al., 2014).

The effector function of antibodies can be further enhanced by post-transcriptional modifications, including glycosylation of the Fc portion. Such modifications improve the ability of the antibody to interact with Fc- and complement-receptors and deposit antigen in the form of immune complexes in the germinal centre (Lux et al., 2013; Nimmerjahn and Ravetch, 2008; Phan et al., 2009). In doing so, it is thought that glycosylated antibodies potentiate germinal centre reactions and drive affinity maturation, therefore leading to enhanced production of nAb (Chung et al., 2015; Lofano et al., 2018). Manipulation of the Fc portion thus represents a promising angle in the development of vaccines to improve the neutralising activity of antibodies.

Finally, it is worth considering that nAb are not preferentially selected for in the immune response. In contrast, antibodies are selected by virtue of the ability of the B cell to capture and present viral antigens. As a result, only a small fraction of virus-specific memory B cells make nAbs. For classic viral infections or therapeutic vaccination, having a variety of antibodies with a range of different effector functions is key.

1.4.7 Effector functions of B cells – beyond antibodies

1.4.7.1 Cytokine production

Through the secretion of cytokines, B cells are able to guide the development of lymphoid tissues, shape T cell responses and negatively regulate immunity. Early in the development of lymphoid tissue, lymphotoxin-α1β2 (LTα1β2) -expressing B cells actively secrete tumour necrosis factor (TNF), required for the development of follicular dendritic cells and the subsequent organization of the B cell follicle (Shen and Fillatreau, 2015). During viral infection, expression of LTα1β2 by B cells is responsible for the expansion of the lymph nodes and germinal centre response, implicating a direct role for B cell cytokine production in the generation of memory B cell responses (Kumar et al., 2010).

LTα1β2, IL-2 and IL-17 expression by B cells has also been demonstrated to directly regulate Th2-type responses in bacterial and parasitic infections (reviewed in (Shen and Fillatreau, 2015)). More relevant to viral infection however is the production of pro-inflammatory cytokines and the promotion of Th1-type responses by B cells. Following engagement of the BCR and CD40, B cells produce TNFα, IL-6 and lymphotoxin (Duddy et al., 2004). Indeed, some of the first evidence describing the ability of B cells to produce pro-inflammatory cytokines demonstrated that B cell depletion ameliorated symptoms of disease in treatment of autoimmune diseases, independent of changes in autoantibody titres (Barr et al., 2012; Sanz et al., 2007). IL-6 production by B cells is induced rapidly upon influenza infection and drives IL-21 expression in CD4 T cells, thus promoting their differentiation to T_{FH} cells and driving the initiation of antiviral immune responses (Karnowski et al., 2012). Similarly, absence of B cell-derived IL-6 decreased the formation of T_{FH} cells, resulting in fewer GC B cells and a reduction in class-switched antibody (Arkatkar et al., 2017). Combined, these data suggest that B cell derived IL-6 is important in the formation of germinal centre responses and the generation of humoral immunity.

Through the production of immunosuppressive cytokines, B cells also have a role in the suppression of adaptive immune responses, predominantly mediated through secretion of IL-10. A major population of IL-10-producing B cells has been defined as B regulatory cells (Bregs). These cells have a role in suppressing immunopathology by prohibiting the expansion of pathogenic T cells and other pro-inflammatory cell types (Rosser and Mauri, 2015). This effect is predominantly mediated by the biasing of T cell responses away from Th1 responses and towards functionally suppressive regulatory T cells (Flores-Borja et al., 2013). As such, numerical and functional deficiencies in Bregs are associated with worse outcomes in autoimmune diseases, including SLE, RA and MS (Mauri and Menon, 2017; Rosser and Mauri, 2015).

Bregs have also been demonstrated in the context of chronic viral infection whereby expansion of IL-10-producing cells with regulatory phenotypes suppresses protective antiviral responses (Dai et al., 2017). In particular, IL-10^{hi} Bregs are increased in patients with HIV and correlate with markers

of disease progression and exhausted T cell responses (Siewe et al., 2013). In CHB, the frequency of Bregs is associated with hepatic flares; an expansion of Bregs suppressed antiviral CD8 T cell responses in an IL-10-dependent manner (Das et al., 2012), discussed in more detail later. IL-10 production has also been demonstrated within additional B cell subsets, including plasmablasts and plasma cells. For example, IL-10 production by plasmablasts was shown to limit inflammation in experimental autoimmune encephalomyelitis (EAE), whilst IL-10-producing plasma cells suppressed inflammatory responses during EAE and *Salmonella* infection (Lino et al., 2018; Matsumoto et al., 2014; Shen and Fillatreau, 2015).

Thus, B cells have important roles in adaptive immune response beyond the production of antibodies; throughout their progression towards terminally differentiated plasma cells, B cells have the potential to modulate immune responses through the types of cytokines they secrete.

1.4.7.2 Antigen presentation

In addition to cytokine production, B cells are also capable of presenting antigen whereupon they can prime CD4 T cell responses (section 1.4.2.2) (Chen and Jensen, 2008; Rodríguez-Pinto, 2005). Crosslinking of the BCR by antigen results in B cell activation, growth and survival (Ackermann et al., 2015; De Silva and Klein, 2015). Antigen-activated B cells upregulate expression of co-stimulatory molecules, CD80 and CD86, required for naïve T cell activation. Activated B cells that have received T cell help via CD40-ligation demonstrate enhanced antigen processing and presentation, imparted by increased expression of MHC Class-II molecules and stabilisation of CD80/86 expression (Roy et al., 1995; Wu et al., 1995).

In vivo, antigen-specific B cells form cognate interactions with T cell responses (Lanzavecchia, 1985) allowing antigen-specific B cells to act as professional antigen presenting cells (APC) during initial CD4 T cell priming and the activation/maturation of T_{FH} cells in the germinal centre. However, as antigen-specific B cells represent only a low frequency of cells prior to immunisation, it currently thought that B cells minimally contribute to the initial activation of naïve CD4 T cells. Moreover, the degree to which B cells participate in T cell priming is thought to be dependent on the type and form of antigen used, and as such has been shown to vary significantly according to the setting (Constant et al., 1995; Macaulay et al., 1998; Rodríguez-Pinto, 2005). However, the antigen-presenting capacity of antigen-specific B cells represents a favourable target in the development of vaccines against infectious diseases. Recent vaccine trials have shown superior induction of CD4 and CD8 T cell responses following antigen-presentation by B cells, rather than classical APCs, such as dendritic cells (von Bergwelt-Baildon et al., 2002; Hong et al., 2018). Despite existing at a lower frequency than DCs, the capture of antigens through specific, high-affinity BCR binding is thought to enhance the amount of antigen that can be presented and thus provide stronger TCR stimulation.

1.5 B cells and neutralising antibodies in the control of persisting viruses

B cells predominantly contribute to the control of persistent viral infections through the production of antibodies that neutralise the virus. Natural HIV or HCV infection elicit the production of affinity-matured class-switched antibodies that target viral proteins but fail to control the disease. However, between 10 and 25% of patients infected with HIV develop antibodies that are capable of neutralising virus isolates. These are detectable ~3-12 months following infection (Hraber et al., 2014; Landais et al., 2016; Li et al., 2009) whereupon they exert a strong selection pressure for HIV to mutate and evade specific antibody responses (Richman et al., 2003; Wei et al., 2003). As a result, these antibodies tend not to supress viral replication in infected patients, but can contribute to sustain low viral loads in some individuals (Bonsignori et al., 2014). Their role in the control of HIV was elegantly demonstrated in a case study where an HIV-infected patient received Rituximab for the treatment of low-grade lymphoma. B cell depletion resulted in a decrease in nAb titres and a concomitant rise in HIV viral load (Huang et al., 2010). Decreased prevalence of nAbs facilitated an increase in genetic diversity of the virus, which in turn led to a transient decrease in the neutralising activity of resulting antibodies. Of note, this study also illustrated the effect of Rituximab on triggering HBV reactivation in HBV-resolved individuals, discussed in more detail in section 1.6.2.

Hence, the most efficient antibody responses are those that can detect a variety of viral isolates and allow the humoral response to keep pace with viral diversification, termed broadly neutralising antibodies (bnAb). Approximately 1% of HIV seropositive individuals develop cross-reactive nAbs that are capable of targeting hundreds of different viral isolates (Simek et al., 2009). These antibodies have provided a target for the development of vaccines and therapeutic monoclonal antibodies (mAb) that aim to supplement or replace humoral responses during, or prior to, viral infection.

Therapeutic vaccination at the time of primary infection or early in the course of infection has been proposed as a method to limit establishment of the viral reservoir and promote viral eradication. Whilst preliminary trials testing an HIV therapeutic vaccine have demonstrated induction of nAbs (Ensoli et al., 2015), these have failed to consistently reduce levels of plasma viraemia (Pantaleo and Levy, 2016; Sneller et al., 2017). Instead, passive immunisation of mAb, designed to reflect bnAbs, has been proposed as a method of preventing infection and neutralising circulating virus. Careful testing of mAb against a large panel of HIV pseudoviruses has permitted the selection of antibodies that have shown potent abilities in suppressing viraemia and can elicit host immune responses (Bar-On et al., 2018; Caskey et al., 2015; Mendoza et al., 2018). Importantly, by using a combination of two bnAbs, Mendoza et al., were able to demonstrate potent viral suppression in the absence of antiretroviral therapy that prevented the development of resistant viruses (Mendoza et al., 2018).

Likewise, bnAbs have shown potent affects in abrogating HCV infection in a human liver chimeric mouse model (de Jong et al., 2014). bnAbs with the capacity to neutralise diverse HCV strains can

be detected and isolated from HCV-infected individuals, despite the large genetic diversity of HCV (Giang et al., 2012; Hadlock et al., 2000; Keck et al., 2012; Kong et al., 2012; Merat et al., 2016). Control of HCV infection is associated with the development of broadly neutralising antibody responses that target the viral envelope proteins (Osburn et al., 2014). While it is possible to detect nAbs in the serum of patients with chronic HCV, these antibodies fail to control infection, likely due to repeated mutational change resulting in loss of recognition (von Hahn et al., 2007).

Isolation of nAbs from patients that spontaneously clear HCV has identified critical epitopes that favour early selection and development of bnAbs during infection, providing putative targets to guide the development of immunogenic vaccines (Bailey et al., 2017). Recent work has demonstrated that different combinations of nAbs may alter the efficacy of these approaches. It showed that using certain combinations of nAbs could increase their potency by simultaneously targeting three different HCV receptors (Mankowski et al., 2018), suggesting that vaccine development should aim to induce multiple nAbs with distinct neutralisation profiles. These paradigm-changing findings have reignited interest in understanding how nAbs develop and the types of B cells required for antibody-mediated viral control.

1.5.1 Viral subversion of humoral immune responses

As alluded to previously, the extensive co-evolution of viruses and hosts has resulted in the fruition of a number of defence strategies employed by viruses to evade protective antibody responses. In this way, viruses have been postulated to interfere with multiple steps of early B cell activation and differentiation, resulting in negligible or low-level polyclonal antibody responses (Kuka and Iannacone, 2017). Viruses can also have direct cytopathic effects on B cells through infection via the BCR or specific cellular receptors. This results in the elimination of early antigen-specific B cells, thus interrupting early antibody responses and maximising viral transmission, as demonstrated by influenza A virus (Dougan et al., 2013). Similarly, virus infection of B cells can modulate the proliferative capacity of B cells and perturb their differentiation. Early studies investigating infection of B cells by measles virus or cytomegalovirus, demonstrated the ability of these viruses to suppress proliferation and differentiation of B cells into antibody-secreting cells (McChesney et al., 1986, 1987; Rice et al., 1984).

Viral proteins, such as HIV-derived negative factor protein *Nef*, have also been shown to accumulate within B cells, where they are able to suppress immunoglobulin class-switching through the inhibition of intracellular signalling pathways, including nuclear factor-κB signalling (Qiao et al., 2006). Similarly, engagement of CD81 expressed on B cells via HCV viral proteins induces hypermutation of the immunoglobulin heavy chain, reducing the affinity and specificity of HCV-specific antibodies (Machida et al., 2005, 2008). Collectively, these data demonstrate how viruses

and associated proteins can modulate the formation of high-affinity antibodies and enable viral escape from immune surveillance.

Within the lymph node, virally-induced inflammation disrupts the organisation of macrophages within the SCS, thus reducing the capacity of SCS macrophages to acquire antigen (Gaya et al., 2015). Whilst this may not directly influence short-term responses to antigen, perturbation of the SCS macrophage responses is postulated to affect the generation of humoral responses to secondary infections through impairing the subsequent induction of GC B cells and plasma cells.

Finally, virus-specific CD8 T cells have been shown to hinder humoral immune responses, either through killing of antigen-specific B cells or elimination of antigen-presenting cells. Virally-infected B cells present viral antigens on MHC Class I molecules, and as such become a target for virus-specific cytotoxic T cells (Battegay et al., 1993; Planz et al., 1996). Similarly, non-infected B cells can also become targets for cytotoxic deletion through the cross-presentation of exogenous viral antigens, as previously demonstrated in HBV infection (Barnaba et al., 1990).

Other recognised mechanisms adopted by viruses to evade antibody responses include antigenic mutation and the induction of B cell anergy and exhaustion, discussed in more detail in Chapter Four.

1.6 B cell responses in CHB

1.6.1 Humoral immunity in HBV

The kinetics of HBV-specific antibody responses can be used to distinguish different stages of HBV infection (Gerlich, 2013). Polyclonal antibodies generated by HBV infection are directed against a range of viral antigens, including HBcAg, HBeAg and the large, middle and small forms of HBsAg. HBcAg-specific IgM (anti-HBc) appears early on in infection and can co-exist with a high level of HBV replication (Hoofnagle et al., 1973). Thus, the detection of anti-HBc is one of the first indicators of active HBV infection and is the only confirmatory marker of previous or current infection (Wang et al., 2017). The immunogenicity of anti-HBc responses is postulated to derive, in part, from the capacity of B cells to present HBcAg. Murine B cells were demonstrated to display enhanced receptor-mediated uptake of HBcAg, compared to professional APCs, that was required for efficient priming of naïve T cells and antibody production following immunisation with HBcAg (Milich et al., 1997). Following this, it was later found that B cells are also required for priming of cytotoxic T cell responses to exogenous, but not endogenous, particulate HBcAg (Lazdina et al., 2003). Thus, as a consequence of its potent immune stimulatory properties, HBcAg has been proposed as a powerful carrier and basis for vaccines against less immunogenic antigens (Whitacre et al., 2009).

In contrast to HBcAg, antibodies specific for HBsAg and HBeAg appear much later, and are associated with favourable outcome of the infection. Antibodies against HBeAg are regarded as a predictor of long-term clearance of HBV, with seroconversion commonly associated with normal serum ALT levels and low levels of HBV DNA (<2000IU/ml) (Gill and Kennedy, 2015). Following successful control of the virus, HBsAg is lost from the blood and HBsAg-specific antibodies (anti-HBs) become detectable. Thus, anti-HBs are considered to be protective and associated with resolution of disease.

Generally, measurements of HBV-specific antibodies have been used in the serological definition of disease phases and functional cure, and their role in immune control has been little studied. However, two classes of anti-HBs antibodies have demonstrated neutralising activity. Until the recent discovery of the HBV entry receptor, NTCP, the ability of anti-HBs antibodies to confer protection was poorly understood. Precise mapping of NTCP facilitated identification of HBV regions essential for infectivity, namely the pre-S1 domain and the a-determinant of HBsAg (Figure 1.7). The first class of neutralising antibody (nAb) described recognises the a-determinant region of the antigenic loop of HBsAg, and is thus capable of blocking interactions with the HSPG (Sureau and Salisse, 2013) and inhibiting secretion of HBsAg from infected hepatocytes (Neumann et al., 2010; Schilling et al., 2003). A second class of antibodies recognises the NTCP-binding site of the Pre-S1 (Ni et al., 2014; Yan et al., 2012), preventing *de novo* infection of hepatocytes (Yan et al., 2012). In contrast, antibodies specific to other HBV regions not involved in HBV infection, such as

the Pre-S2 region, appear to have no relevance to natural HBV immunity (Hellström et al., 1986; Ni et al., 2010; Seyec et al., 1998).

Hence, vaccine-induced production of anti-HBs has an important role in pre-exposure prophylaxis and protection against *de novo* infection. Once established through vaccination, immunity against HBV is usually maintained for life, despite waning levels of anti-HBs (McMahon et al., 2009). The latest generation of vaccines, that include epitopes for Pre-S1/2, have shown enhanced immunogenicity, resulting in faster and higher rates of seroconversion. Importantly, these vaccines also result in lower rates of non-responders and are less affected by extraneous variables, including body weight (Krawczyk et al., 2014; Shouval et al., 2015).

The neutralising capacity of anti-HBs antibodies is underscored by their ability to block HBV infection (Cerino et al., 2015; Glebe et al., 2003), and has been demonstrated in primate models, whereby inoculation of chimpanzees with human mAbs prior to viral challenge prevented the development of infection (Hong et al., 2004; Kim et al., 2008). Accordingly, transfer of antibodies directed against HBV proteins can have protective effects in the settings of liver transplant and post-exposure prophylaxis in neonates born to HBsAg-positive mothers (Beasley et al., 1981; Shouval and Samuel, 2000). In settings of liver transplantation, polyclonal hepatitis B immunoglobulins, isolated from the plasma of HBV-vaccinated or HBV-resolved healthy donors, is routinely administered prior to and repeatedly following surgery to maintain protective levels of anti-HBs and prevent HBV recurrence (Varghese et al., 2014).

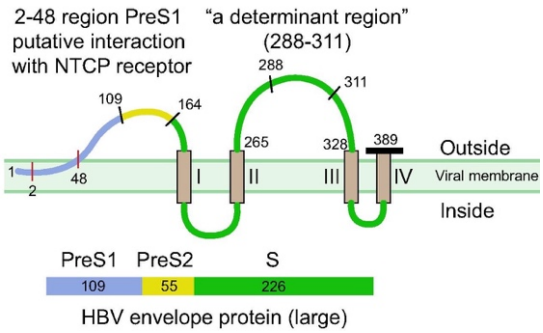

Figure 1-7 Structure of HBsAg

Schematic representation of the conformation of HBsAg, showing the PreS1 region and a determinant region that interact with neutralising antibodies [Adapted from (Bertoletti and Ferrari, 2016)]

Current efforts are now focused on alternative strategies of pre-exposure and post-exposure prophylaxis, including the development of mAbs against HBsAg or Pre-S1, similar to those identified in HIV (Corti et al., 2018). Numerous mAbs have now been developed that show potent neutralising activity (Cerino et al., 2015; Kucinskaite-Kodze et al., 2016; Shin et al., 2007b) including HB-C7A, which possesses the capacity to prevent HBV infection in chimpanzees (Kim et al.,

2008). This mAb is now in clinical trials to evaluate its efficacy in protecting against infection following liver transplantation, and in the event of HBV exposure.

In addition to their capacity to block viral entry via Fab recognition of virions, mAbs can also act *in vivo* through Fc-dependent mechanisms. Murine studies using novel nAbs targeted against the a-determinant (mAb E6F6 (Zhang et al., 2015)) or Pre-S1 region (mAb 2H5-A14 (Li et al., 2017)) significantly reduced the levels of HBsAg and HBV DNA in HBV-transgenic mice through Fc-dependent mechanisms. The suppressive abilities of these antibodies were inhibited when Fc-receptor binding was impeded through mutation of the Fc portion. Administration of E6F6 in transgenic mice promoted viral clearance and blocked viral infection, dependent on Fc-mediated phagocytosis; when the Fc-portion was mutated, less effective suppression of HBV was observed. Similarly, 2H5-A14, but not its Fc-mutated variant, significantly reduced levels of cccDNA in HBV-infected mice. HBV suppression was mediated through sequestration of viral and sub-viral particles by antibody-dependent cellular phagocytosis (ADCP), in addition to NK cell-mediated killing of infected hepatocytes (ADCC). Human mAbs have shown promise in reducing HBsAg to undetectable levels in patients with CHB, corresponding with a decrease in HBV DNA (Galun et al., 2002). Despite being well tolerated, monotherapy was insufficient to maintain low levels of antigen and virus. Alternative approaches proposed include clearance of extracellular antigen via inoculation with nAbs, followed by prophylactic vaccination to restore protective HBsAg-specific CD4 and B cell responses (Zhu et al., 2016). Combined, these data suggest that antibodies targeting HBsAg may provide an important arm of combination therapies in patients with CHB. Currently, numerous groups are working on developing therapeutic nAbs through the characterisation of anti-HBs produced by virus-controllers.

Despite evidence showing that anti-HBs is protective and can have neutralising properties, robust data regarding the frequency of nAb-producing cells during CHB are lacking. However, the identification of antibodies that can block HBV viral entry presents the possibility that neutralising anti-HBs may play a role in modulating the development of persistent infection through blocking HBV spread to non-infected hepatocytes. The ability of HBV to spread via NTCP was clearly demonstrated in murine models of HBV infection, where blockade of NTCP with Myrcludex B peptide prevented HBV spread in infected livers (Petersen et al., 2008). Lack of detectable anti-HBs in patients with CHB would suggest that B cells producing nAb are numerically or functionally defective. As such, humoral immunity in CHB may be improved through thorough characterisation and targeting of anti-HBs-producing cells.

1.6.2 Evidence for a role of B cells in the immune control of CHB

Relatively little is known pertaining to the role of B cells in the persistence of CHB. Patients with CHB are characterised by a failure to seroconvert against the envelope protein of the virus (HBsAg) with detection of antibodies against HBsAg (anti-HBs) regarded as the hallmark of functional cure. To date, few studies have specifically addressed the potential for defective B cell responses to underpin the failure of patients with CHB to undergo HBsAg seroconversion.

Patients with CHB do not have global defects in antibody production and reportedly display an increased frequency of activated global B cells of memory or naïve phenotype that are functionally intact (Oliviero et al., 2011; Xu et al., 2015). Despite showing impaired proliferative capacity relative to healthy controls, B cells retain the ability to differentiate into plasma cells and can robustly produce global immunoglobulin upon simulation (Oliviero et al., 2011). Furthermore, gene expression profiling of peripheral B cells in HBV-infected patients of varying clinical and virological phases of disease, identified a gene-signature consistent with robust B cell activation in patients with chronic active hepatitis (characterised by high ALT levels and viral load) (Vanwolleghem et al., 2015). Although the functional consequences of these studies are as yet unclear, at the very least they provide evidence for HBV infection modulating B cell activity.

The importance of the B cell response in CHB is underscored by the clinical observation that B cell depletion by anti-CD20 or anti-CD52 antibody therapies in the management of lymphoma can lead to the risk of HBV-reactivation in HBV-resolved patients (reactivation determined by the reappearance of HBsAg and HBV DNA in previously negative patients) and $HBsAg^+$ inactive carriers (Huang et al., 2010; Loomba and Liang, 2017; Shouval and Shibolet, 2013). Rituximab depletion in patients prior to kidney transplantation showed a direct relationship between the administration of rituximab and HBV reactivation (Lee et al., 2017). The risk of HBV reactivation has been associated with both host and viral factors, including the genotype of the infecting strain, mutations within the core promoter region and HBV serological markers (Borentain et al., 2010). Of note, meta-analysis of cases of HBV-reactivation in resolved patients showed some protection conferred by existing levels of detectable levels of anti-HBs (Paul et al., 2017). As plasma cells are not depleted by Rituximab, these observations suggest that viral control is mediated by B cell populations that express CD20. Furthermore, it is theorised that Rituximab is not able to deplete B cells from within lymphoid tissue (Hammarlund et al., 2017; Kamburova et al., 2013; Mamani-Matsuda et al., 2008), suggesting that formation and function of HBsAg-specific B cells within lymphoid tissue is not sufficient for adequate control of HBV infection. Crucially, evidence demonstrating suppression of B cell immunity leading to HBV reactivation provides key evidence that B cells may play a previously unappreciated role in ongoing HBV immune control, not just prevention of *de novo* infection.

Previous work from our group has also identified an expansion of circulating and intrahepatic IL-10-producing Bregs, typified by high expression of CD24 and CD38, that correlated with serum levels of IL-10 in patients with CHB undergoing spontaneous disease flares. Co-culture of $CD24^{hi}CD38^{hi}$ immature B cells suppressed HBV-specific CD8 T cells in an IL-10-dependent manner; blockade of IL-10 significantly boosted exhausted HBV-specific CD8 T cell functionality, suggestive of a role for Bregs in suppressing functional T cell responses in CHB (Das et al., 2012).

1.6.3 HBV-specific B cell responses

Production of anti-HBs is generally considered T cell dependent (Milich and McLachlan, 1986) with HBsAg-primed CD4 T cells shown to be essential for anti-HBs production (Wang et al., 2018b). However, possible deficiencies in CD4 T cells cannot solely explain defects in anti-HBs production; co-culture of B cells from patients with CHB with T cells from HBsAg-vaccine responders failed to induce anti-HBs production, pointing to a B cell-specific defect (Barnaba et al., 1985). Moreover, anti-HBs production is unlikely to be completely impaired in all patients with CHB, as demonstrated by the clinical observation of anti-HBs:HBsAg immune complexes in a subset of patients with CHB (Madaliński and Bragiel, 1979) and the capacity to measure low level anti-HBs in some patients with CHB. These data also point to the existence of HBsAg-specific B cells, at least in a subset of patients with CHB (Gerlich, 2007).

As reported for HBV-specific T cells, frequencies of anti-HBs B cells have been shown to be decreased in the periphery of patients with CHB relative to those with acute-resolving infection (Barnaba et al., 1985; Böcher et al., 1999; Dusheiko et al., 1983; Wang et al., 2015; Xu et al., 2015). One potential mechanism for their depletion was proposed to be the deletion of B cells capable of cross-presenting HBsAg on MHC-Class I by cytotoxic T lymphocytes (Barnaba et al., 1990). However, these studies have traditionally relied on functional readout of anti-HBs production for quantification of HBsAg-specific B cells and therefore cannot distinguish between functional or numerical defects in HBsAg-specific B cell responses (discussed in more detail in Chapter Three).

Furthermore, the enumeration of anti-HBs producing B cells in the periphery might not be fully informative. During infection, memory B cells and plasma cells home to inflamed tissues sites and to bone marrow, allowing them to efficiently contribute to the immune response at the site of infection (Kunkel and Butcher, 2003). B cells capable of producing antibodies against the core protein, HBcAg, have been shown to localise to the intrahepatic environment during HBV acute liver failure where they are postulated to drive liver pathology (Farci et al., 2010). $CD20^{+}$ B cells can also infiltrate the liver vasculature and parenchyma, although the function of these cells in CHB is as yet unknown (Mohamadkhani et al., 2014). Further investigations are required to address the possibility that HBsAg-specific B cells can localise to the liver and play a role in the local immune response (discussed in more detail in Chapter Five).

1.7 Thesis hypothesis

Patients with chronic HBV infection are characterised by their failure to produce detectable protective responses to the HBV envelope protein, HBsAg. Due to their emerging role in the control of viral infections through the production of neutralising antibodies and cytokines, it was thought that reduced antiviral B cell function may contribute to the persistence of HBV. This concept is underpinned by the key finding that Rituximab depletion of $CD20^+$ B cells can trigger HBV reactivation in functionally-resolved patients, and worsening of disease in patients with chronic infection, suggesting that B cells support viral control.

Specifically, it was hypothesised that the high HBsAg-load in patients with CHB may result in defects in the frequency and function of HBsAg-specific B cells that underlie their inability to form protective anti-HBs responses.

1.8 Thesis aims

- To determine whether frequencies of HBsAg-specific B cells are decreased in patients with CHB relative to those with protective immunity;
- To determine whether HBsAg-specific B cells in patients with CHB are capable of producing protective anti-HBs, implicated by the lack of anti-HBs production in patients' serum;
- To characterise HBsAg-specific B cells directly *ex vivo* from patients with CHB to identify putative targets for boosting anti-HBs responses in patients with CHB;
- To assess the possibility that intrahepatic global and antigen-specific B cells localise at the site of viral replication.

1.9 Thesis highlights

1. HBsAg-specific B cells persist at a similar frequency in patients with CHB, compared to those with acute-resolving infection and HBV-vaccine induced immunity;
2. HBsAg-specific B cells from patients with CHB exhibit defective production of protective anti-HBs;
3. HBsAg-specific B cells demonstrate an accumulation of atypical memory B cells (atMBCs), characterised by high levels of inhibitory receptor expression and impaired antiviral function;
4. HBsAg-specific B cells can be identified within HBV-infected liver yet show similar enrichment of dysfunctional B cell subset.

Chapter 2 Materials and Methods

2.1 Study participants

Samples utilised within this study were obtained from patients attending clinics at four separate sites across London:

- Mortimer Market Sexual Health Clinic – Central and North-West London NHS Foundation
- Royal Free Hospital
- University College London Hospital (UCLH)
- Royal London Hospital – Barts Health NHS Trust

This study was approved by the local ethical boards of London-Brent (REC number 16/LO/1699) and Brighton and Sussex (REC number 11/LO/0421). Each participant gave written informed consent. All storage of samples obtained complied with the requirements of the Data Protection Act 1998 and the Human Tissue Act 2004.

Samples from a total of 118 patients with CHB and 6 patients with HCV were used within this study. For comparison to HBV-naïve controls, 74 age and sex matched healthy volunteers were recruited from staff, students, and friends of University College London. A separate cohort of 24 HBV-unvaccinated healthy controls was collected for the purpose of confirming negativity of HBsAg-specific B cells in HBV-naïve controls.

All patients with CHB were anti-HCV and anti-HIV antibody negative and treatment naïve, unless otherwise stated. Patients were stratified by a number of clinical disease parameters throughout the study. These were obtained as part of routine diagnostic assessments by the relevant NHS laboratories and include the following:

1. HBeAg status (defined as either positive or negative)
2. HBV viral load (IU/ml, determined by real-time polymerase chain reaction)
3. Levels of HBsAg (IU/ml, Abbott Architect Quantitate HBsAg)*
4. Biochemical evidence of liver inflammation, determined by serum levels of alanine transaminase (IU/L)
5. Anti-HBs (defined as either positive or negative)

*Where unavailable, assessments were made in-house using highly sensitive chemiluminescent immunoassays, described in more detail in section 2.6.3

The full sample cohort is detailed in Table 2.1.

2.1.1 HBV acute-resolving patients

Eight patients were identified as having acute HBV infection upon presentation in the clinic and were tracked longitudinally throughout resolution of disease. Acute HBV infection was diagnosed on the basis of new HBsAg positivity or recent exposure, and serological evolution (i.e. detection of anti-HBc IgM/IgG). Samples were considered from the acute phase when HBV DNA was still detectable and ALT was greater than 40IU/L. Patients were identified as resolved when HBV DNA and HBsAg were undetectable and ALT levels were normalised (below 40IU/L). Longitudinal acute-resolved samples are numbered and referred to consistently throughout.

2.1.2 Intrahepatic samples

Resected liver samples from the healthy margins of colorectal metastatic tumour resections were obtained through the Tissue Access for Patient Benefit scheme at The Royal Free Hospital (approved by the University College London Royal Free Hospital BioBank Ethical Review Committee; Research Ethics Committee reference number 11/WA/0077). Perfusion liquid was obtained from healthy livers prior to solid-organ transplantation (RIPCOLT clinical trial; Research Ethics Committee reference number 11/H0720/4; trial number 8191; trial registered at clinicaltrials.gov: NCT00796588).

For HBV^+ liver, tissue was obtained from liver biopsies deemed surplus to diagnostic requirements from 10 patients with treatment-naïve CHB, attending clinics at The Royal London Hospital (approved by East London and The City Research Ethics Committee; Research Ethics Committee reference number P/01/023). Four additional HBV^+ liver samples were obtained from explanted livers with end-stage HBV-associated disease (Tissue Act for Patient Benefit Scheme)

Full details of liver samples used are detailed in Table 2.2.

Table 2-1 Full details of study participants (PBMCs)

Abbreviations used: below the level of quantification (blq); serum alanine transaminase (ALT); not applicable (N/A); hepatitis B surface antigen titre (HBsAg); hepatitis B secreted antigen titre (HBeAg)

	Age (years) median (range)	**Sex (%)** male:female	**ALT (IU/L)** median (range)	**Viral load (IU/ml)** median (range)	**HBsAg (IU/ml)** median (range)	**HBeAg (%)** pos:neg
CHB (n=118)	41 (24-75)	64:36	36 (13-297)	3.1×10^4 (blq - 1.1×10^9)	6370 (108 – 3.6×10^6)	26:74
HC (n=74)	33 (20-84)	50:50	N/A	N/A	N/A	N/A

Table 2-2 Full details of intrahepatic samples

	Age (years) median (range)	**Sex (%)** male:female	**ALT (IU/L)** median (range)	**Viral load (IU/ml)** median (range)	**HBsAg (IU/ml)** median (range)	**HBeAg (%)** pos:neg
HBV-infected (n=14)	41.5 (32–66)	69:31	47 (25-390)	5567 (blq – 6.0×10^7)	7676 (0 - 21275)	23:77
CRC resections (n=22)	65 (23-80)	71:29	257 (17-1013)	N/A	N/A	N/A
Healthy perfusates (n=12)	46 (16-66)	63:37	N/A	N/A	N/A	N/A

2.2 Sample isolation and preparation

2.2.1 Preparation of Peripheral Blood Mononuclear Cells (PMBC)

Venous blood samples were collected in sterile 9ml BD Vacutainers® containing lithium-heparin and processed immediately. Peripheral blood mononuclear cells (PBMCs) were isolated by density centrifugation using Ficoll® Paque Plus (GE Healthcare). Whole blood was diluted in RPMI 1640 (GIBCO™) and layered at a 2 : 1 ratio on Ficoll® Paque Plus, followed by centrifugation at 2200 rpm at 30°C for 22 min with minimum acceleration and brake. PBMCs were carefully extracted from the interface using Pasteur pipettes and washed twice via dilution with RPMI 1640 and centrifugation at 1800 rpm for 15 min. Cell counts were determined using a Neubauer counting chamber under light-microscopy; to count, cells were first diluted in RPMI 1640, then 1 : 1 in trypan blue (a viability dye that stains the nucleus of dead cells). The number of cells was determined using the following formula:

$$N = M \times D \times 10^4$$

N = number of cells/ml
M = number of cells counted in grid of 16 squares (taken as an average of 3 grids)
D = dilution factor

PBMCs were either used immediately or frozen in foetal bovine serum (Invitrogen) supplemented with 10% DMS0 (Sigma-Aldrich).

2.2.2 Freeze/thawing of PBMC

Isolated cells not used directly for experiments were re-suspended at 5×10^6 cells/ml in heat-inactivated foetal bovine serum (FBS) (Invitrogen™) supplemented with 10% dimethylated sulfoxide (DMSO) (Sigma-Aldrich®). Cells were aliquoted in cryovials (Corning) and stored in freezing containers containing isopropanol (Mr Frosty™, Thermo Scientific™) at -80°C for a minimum of 24 h. As soon as possible, cells were transferred for long-term storage in gas-phase nitrogen tanks. When required, PBMC were thawed rapidly at 37°C and washed by centrifugation in 15ml of RPMI 1640.

2.2.3 Preparation of serum samples

During sample collection, additional blood was collected in BD Vacutainers® containing no anti-coagulant. This sample was left for a minimum of 30 min at room temperature (RT) before centrifugation at 1800 rpm. Serum was removed, aliquoted into cryovials, and stored at -80°C for later use.

2.2.4 Preparation of intrahepatic lymphocytes (IHL)

HBV+ infected liver biopsies: IHL from CHB liver biopsies deemed surplus to diagnostic requirement were isolated by mechanism disruption. Biopsies were re-suspended in RPMI 1640 and macerated using a cell-scraper in small Petri dishes. The resulting single-cell suspension was filtered through 70 μm cell strainers to remove debris and prevent cell clumping. Isolated cells were diluted in RPMI 1640 and washed by centrifugation at 1800 rpm at 22°C for 15 min.

Healthy perfusates samples: To isolate IHL from perfusate samples, liquid was first concentrated by centrifugation at 1800 rpm at 22°C for 15 min. Concentrated cells were re-suspended in RPMI 1640 and IHL isolated by density centrifugation using Ficoll® Paque Plus (GE Healthcare), as with PBMCs.

Explants, including margins from colorectal metastases (CRC): With larger explant tissues, sections were cut into small pieces in specimen pots using sterilised scissors, and incubated at 37°C for 30 min in 0.01%-collagenase IV (Thermo Fisher Scientific™) and 0.001%-DNase I (Sigma-Aldrich®). Mechanical digestion was performed using a gentleMACS™ tissue dissociator (Miltenyi Biotec®). Single-cell suspensions were filtered as before, before dilution in HBSS (Gibco®) and centrifugation 1800 rpm at 22°C for 15 min. Cells were re-suspended in 30%-Percoll® (Sigma-Aldrich®) diluted in HBSS (Gibco®), and centrifuged at 2000 rpm at 22°C for 10 min. Following centrifugation, parenchymal cells were removed using a Pasteur pipette. The remaining pelleted cells were then diluted in RPMI 1640 and IHLs isolated by density centrifugation using Ficoll® Paque Plus (GE Healthcare), as before.

In all instances, IHL were counted as before or using an automated cell counter (ADAM-MC™) that provides a count of viable cells with a higher degree of accuracy. In all instances, IHL were used directly for *ex vivo* experimentation and characterization.

Liver-draining lymph node from one patient with HBV-associated end-stage liver failure was processed as per HBV-liver biopsies.

2.3 Multi-parametric Flow Cytometry

All antibody staining for flow cytometry was performed on a single-cell suspension either in 96-well pates (U-bottomed) or in 5ml polypropylene tubes.

2.3.1 Surface staining of PBMC and IHL for B cell frequency and phenotype

To determine B cell frequencies and phenotypes, a minimum of 2×10^6 cells was used. Cells were initially stained with a fixable viability dye (LIVE/DEAD™, Invitrogen™) for 15 min at 4°C, to permit identification and removal of dead cells during analysis. Following staining, the dye was washed off using 1× PBS and centrifugation at 1800 rpm for 4 min at 4°C. Cells were blocked to prevent unwanted binding of antibody to Fc-receptor-expressing cells (FcR blocking reagent, Miltenyi Biotec®) for 15 min at 4°C. Samples were stained in the presence of FcR blocking reagent using saturating concentrations of relevant directly-conjugated anti-human mAbs for 30 min at 4°C in the dark. Antibodies were diluted in 50%-Brilliant Stain buffer (BD Horizon™, BD Biosciences™) and 50%-PBS to minimize interactions between multiple fluorescent dyes. Appropriate isotype controls were used where necessary. Cells were washed in 1× PBS and fixed with BD Cytofix/Cytoperm™ (BD Biosciences™). All samples were acquired on either an LSRII or Fortessa-X20 flow cytometer (BD Biosciences™) immediately or the following day, and analysed using FlowJo (Tree Star). Full details pertaining to surface mAb used are listed in Table 2.3.

2.3.2 Identification of HBsAg-specific B cells

For identification of HBsAg-specific B cells, a minimum of 1×10^6 cells was stained with an Alexa Fluor 488-conjugated recombinant HBsAg (ayw strain; F. Hoffman-La Roche™). All staining was performed in parallel with mAb staining (as above) and incubated for 30 min at 4°C in the dark. To reduce non-specific staining, mAb were diluted in PBS supplemented with 0.5%-FBS and 2 mM Ethylenediaminetetraacetic acid (EDTA). Stringent gating criteria were applied with doublet, dead, and CD19-negative cell exclusion to minimize non-specific binding contamination. Cells stained with an identical panel minus HBsAg-bait (FMO; fluorescence-minus-one), were used to control for non-specific binding, with corresponding FMO frequencies subtracted. The phenotype of HBsAg-specific B cells was not analysed in instances where there were fewer than 50 HBsAg-bait$^+$ cells recorded.

2.3.3 Flow cytometric staining for intracellular and intranuclear antigens

For the detection of intracellular antigens, cells were blocked and extracellular markers were stained as in section 2.3.1. Once stained, cells were fixed in BD Cytofix/Cytoperm™ (BD Biosciences™) for 20 min at 4°C in the dark. After fixation and permeabilisation, cells were washed and stained with relevant directly-conjugated mAb in the presence of 0.1%-saponin for 30 min at 4°C in the dark. Appropriate isotype controls were used where necessary. After staining, the cells were washed and re-suspended in PBS, before acquisition.

For the detection of intranuclear antigens, cells were blocked and extracellular markers were stained as in section 2.3.1. A minimum of 5×10^6 cells were used. Once stained, cells were fixed and permeabilised using FoxP3 Buffer Set (BD Biosciences™) according to manufacturer's instructions. Briefly, cells were fixed for 10 min in Buffer A before washing and permeabilisation in buffer B for a further 30 min. Cells were washed and then incubated at 4°C for 30 min with relevant directly-conjugated mAb diluted in 1× PBS. After staining the cells were washed and re-suspended in PBS, before acquisition. Full details pertaining to intracellular and intranuclear mAb used are listed in Table 2.3.

Table 2-3 Full details of mAb used

Directly-conjugated anti-human mAbs against extracellular, intracellular and intranuclear antigens for the phenotypic and functional description of B cell subsets

Antigen	Fluorochrome	Clone	Manufacturer	Cat No.	Dilution
Extracellular antigens					
CD45	BUV805	HI30	BD Biosciences™	564914	0.5:100
CD19	BV786	SJ25C1	BD Biosciences™	563325	2:100
CD20	Alexa-Fluor® 700	2H7	BD Biosciences™	560631	1:100
CD3	BV711	OKT3	BioLegend	317328	1:100
CD3	BV510	OKT3	BioLegend	317332	1:100
CD10	BV605	Ber-ACT8	BioLegend	350218	2:100
CD21	BV421	B-Ly4	BD Biosciences™	562996	2:100
CD27	BUV395	L128	BD Biosciences™	561767	1:100
IgM	APC/Cy7	MHM-88	BD Biosciences™	314520	1:100
IgD	PE/Cy7	IA6-2	BD Biosciences™	561314	1:100
IgD	PerCP-Cy5.5	IA6-2	BioLegend	348208	1:100
PD-1	PE	EH12.2H7	BioLegend	329906	2:100
FcRL5	APC	509f6	BioLegend	340306	2:100
CD32b	PE	FUN-2	BioLegend	303205	2:100
BTLA	APC/Cy7	MIH26	BioLegend	344517	2:100
CD22	PE/Cy7	HIB22	BioLegend	302514	1:100
CD80	BV510	L307.4	BD Biosciences™	563084	1:100
CD11c	BV711	3.9	BioLegend	301630	2:100
CXCR5	APC/Cy7	J252D4	BioLegend	356925	3:100
CXCR3	PerCP-Cy5.5	IC6/CXCR3	BD Biosciences™	560832	2:100
CD24	PE/Cy7	ML5	BioLegend	311120	1:100
CD38	PE-dazzle	HIT2	BioLegend	303532	0.5:100
Intranuclear antigens					
T-bet	EFluor666	EBio4B10	eBioscience	50-5825-82	1:100
Ki67	PE-Cy7	20Raj1	eBioscience	25-5699-42	1:100
Blimp-1	PE	6D3	BD Biosciences™	564702	1:100
Bcl-2	Alexa-Fluor® 488	100	BioLegend	658703	2:100
Intracellular antigens					
TNFα	FITC	MAb11	BD Biosciences™	554512	1:100
IL-6	APC	MQ2-13A3	BioLegend	501112	3:100

2.4 B cell functional assays

For functional experiments, cells were cultured in supplemented RMPI 1640 (referred to as complete RPMI and abbreviated to cRPMI) unless otherwise stated. This consisted of the following:

- 10% heat-inactivated FBS (Invitrogen™)
- 100 U/ml penicillin/streptomycin (Invitrogen™)
- MEM essential amino acids (Invitrogen™)
- MEM non-essential amino acids (Invitrogen™)
- Hydroxyethyl piperazineethanesulfonic acid (HEPES; Invitrogen™)
- β-mercaptoethanol (Sigma-Aldrich®)
- Sodium pyruvate (Invitrogen™)

2.4.1 B cell enrichment

PBMCs were thawed and re-suspended in PBS supplemented with 0.5%-FBS and 2 mM EDTA. B cells were enriched by magnetic separation according to manufacturer's instructions (EasySep™ B cell Enrichment Kit, StemCell™ Technologies). Representative examples of the purity achieved using this protocol are shown in Figure 2.1. Enriched B cell populations were used for ELISPOT assays, detection of apoptosis, and calcium flux experiments.

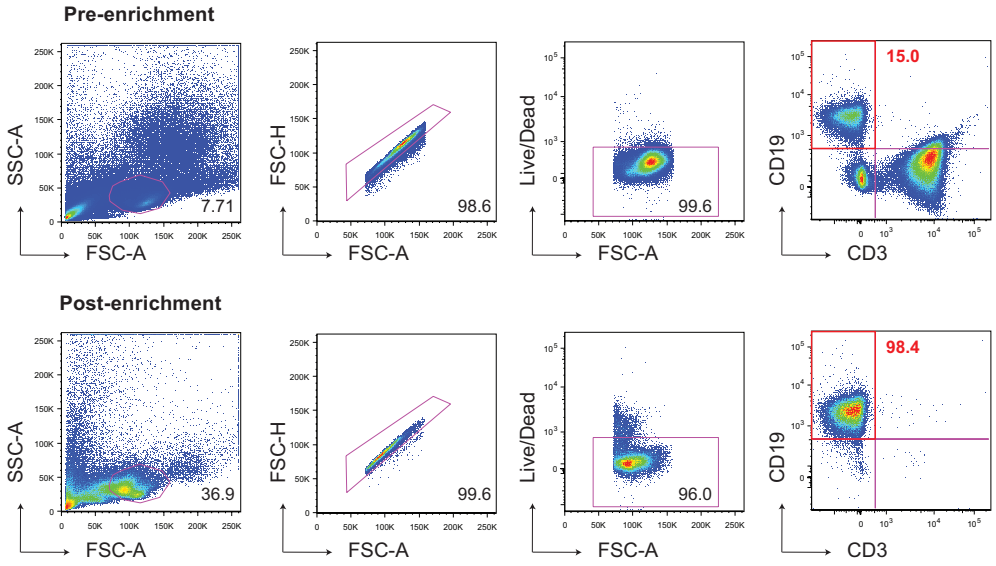

Figure 2-1 B cell enrichment achieved using EasySep™ B cell Enrichment Kit

Representative examples demonstrating the purity achieved following B cell enrichment. Frequency shown represents the frequency of $CD19^+$ cells as a percentage of total live events.

2.4.2 Calcium flux

B cells were separated through magnetic separation according to section 2.4.1 and stained for B cell memory phenotype, as before. Following staining, cells were washed in PBS and incubated with 1 μM of Fluo-4-AM dye (Thermo Scientific™) in the dark for 30 min at 37°C. Fluo-4-AM was diluted in RPMI containing 250 mM probenecid to reduce dye efflux from the cell (Thermo Scientific™). Cells were then washed in PBS and rested in the dark at RT for 20 min before analysis. 5×10^5 cells were analysed for 20 s on a LSR-Fortessa-X20™ (BD Biosciences™) to establish baseline prior to stimulation with anti-IgM/IgG/IgA (50 μg/ml; Jackson ImmunoResearch) or Ionomycin (1 μg/ml). Cells were immediately returned to the machine and fluorescence recorded for a further 150 s. Data were analysed using Kinetics software on FlowJo.

2.5 Phosphoflow

Thawed PBMCs were re-suspended in cRPMI and plated in a 96-well plate prior to staining for cell surface markers to permit identification of memory B cell subsets. mAb were carefully chosen to withstand methanol fixation (detailed in Table 2.4). mAbs was washed off prior to stimulation with anti-IgG/A/M (20 μg/ml; Jackson ImmunoResearch) for 30 s. Cells were immediately fixed in pre-warmed cytofix (BD Biosciences™) and left at 37°C for 10 min. Cells were then pelleted and re-suspended in Phosflow Buffer (BD Biosciences™) for 30 min at -20°C. Following permeabilisation, cells were stained with rabbit monoclonal specific for phosphorylated BLNK (p-BLNK; Cell Signalling Technology®) for 30 min at 4°C. Antibody was washed off, before incubation with PE-secondary (Thermo Scientific™) for a further 30 min at 4°C. Cells were washed and analysed on LSR-Fortessa-X20™ (BD Biosciences™). Unstimulated cells and a stimulated FMO were performed for each individual as gating controls.

Immune complex assays: Recombinant anti-HBs (isolated from single-cell HBsAg-specific B cells; experiments performed by Dr. Laura McCoy) was purified using Protein G beads (Dr. Laura McCoy), and concentrated using Amicon® Ultra Centrifugal Filters (Merck-Millipore) and repeated centrifugation at 3500 rpm. Purified anti-HBs was tested for specificity using standard anti-HBs ELISA and confirmed by UCLH, Health Services Laboratory. Immune complexes were made through incubation of 100 μg/ml of purified anti-HBs with 100 μg/ml of recombinant HBsAg (F. Hoffman-La Roche™) for 2 h at 37°C. To test their effect on BCR signalling, purified B cells were incubated ± 10 μg/ml of immune complexes prior to stimulation, and stained for p-BLNK, as above.

Table 2-4 Cell surface markers for subset identification in phosphoflow assays

Antigen	Fluorochrome	Clone	Manufacturer	Cat No.	Dilution
CD19	eFluro450	HIB19	eBioscience	48-0199-42	2:100
CD20	Alexa-Fluor® 700	2H7	BD Biosciences™	560631	1:100
CD27	FITC	M-5271	BD Biosciences™	555440	1:100
CD3	BV711	OKT3	BioLegend	317328	1:100
CD21	APC	B-Ly4	BD Biosciences™	561767	2:100
p-BLNK (Tyr96)	-	-	Cell Signalling Technology®	#3601	2:100

2.5.1 Detection of B cell cytokine production

To assess cytokine production, PBMCs were rapidly thawed, washed and re-suspended in cRPMI. 2×10^5 PBMCs were stimulated in a 96-well plate for 16-18 h with either 10 µg/ml $F(ab')_2$ anti-IgM and -IgG-specific antibodies (Southern Biotech and Jackson ImmunoResearch, respectively) in combination with 0.5 µg/ml mega-CD40-ligand (Enzo Life Sciences); or 1 µg/ml R848 (Resiquimod; TLR-7/8 agonist; InvivoGen) diluted in complete RPMI (cRPMI). All stimulations were performed in the presence of 1 µg/ml brefaldin-A (Sigma Aldrich®) at 37°C. Unstimulated cells, cultured in cRPMI and BFA only, were used as a negative control. After incubation, cells were washed and cytokine responses analysed by intracellular cytokine staining for IL-6 and TNFα (detailed in Table 2.3), along with cell surface staining to identify memory B cell populations. To assess the effect of PD-1 blockade, cells were stimulated as above in the presence of 10 µg/ml anti-PD-1 mAb or matched isotype control (LEAF™ purified; clone EH12.2H7; BioLegend).

2.5.2 Detection of apoptotic cells – Annexin-V staining

For analysis of apoptosis, cells were stained for Annexin-V *ex vivo*. PBMCs were thawed and cell surface markers stained, as in section 2.3.1. Cells were then washed in Annexin-V binding buffer and incubated with Annexin-V - PerCP/Cy5.5 in the presence of buffer for 15 min, according to manufacturer's instructions (BioLegend). Once stained, cells were washed in PBS and fixed using Cytofix/Cytoperm (BD Biosciences™).

For analysis of apoptosis during culture, 2×10^5 PBMCs were stimulated for 4 d with 1 µg/ml $F(ab')_2$ anti-IgM- and -IgG specific antibodies, plus 500 ng/ml mega-CD40-ligand (ENZO). Cells were then stained for B cell phenotype, followed by incubation with Annexin-V - PerCP/Cy5.5 in the presence of Annexin-V binding buffer for 15 min, according to manufacturer's instructions (BioLegend). To assess the effect of PD-1 blockade, cells were stimulated as above in the presence

of 10 µg/ml anti-PD-1 mAb or matched isotype control (LEAF™ purified; clone EH12.2H7; BioLegend).

2.6 Antibody-secreting cell (ASC) assays

2.6.1 Differentiation protocol

To promote the differentiation of plasma cells, B cells were cultured using a two-step differentiation protocol (Jahnmatz et al., 2013; Jourdan et al., 2009; Walsh et al., 2013). B cells were first activated in a 96-well plate using polyclonal stimulus (CpG-B ODN 2006 InvioGen; 50ng/ml) ± soluble HBsAg (F. Hoffman-La Roche™; ayw, 1 µg/ml).

Step one: Activated cells were expanded using a mixture of B cell homeostatic cytokines, containing IL-2 (50 U/ml), IL-10 (50 ng/ml), IL-15 (10 ng/ml) and IL-21 (100 ng/ml) plus CpG-B (50 ng/ml), diluted in DMEM (supplemented with 10%-FBS, 1%-penicillin/streptomycin and 10%-non-essential amino acids). Cells were incubated for 4 d at 37°C.

Step two: Following activation, cells were switched into DMEM containing IL-2, IL-10, IL-15, (as above), plus IL-6 (50 ng/ml) and IFNα (500 U/ml) and cultured for a further 3 d at 37°C to promote their differentiation.

2.6.2 ELISPOT

ELISPOT plates (multiscreen HTS-IP 0.45µm, Merck/Sigma-Aldrich®) were pre-coated with HBsAg (2 µg/ml), or $F(ab')_2$ anti-IgG (1 µg/ml; Jackson ImmunoResearch) for detection of total antibody-secreting cells, and blocked with RPMI plus 1%-milk. For ELISPOT analysis of HBsAg-specific memory B cells, B cells were purified from frozen PBMC samples using magnetic separation (as in section 2.4.1). Enriched B cells were cultured as in section 2.6.1. At the end of the culture period, cells were washed in cRMPI and counted as in section 2.2.1. For the detection of HBsAg-specific memory B cells, 2×10^5 cells were added to the plate and incubated at 37°C in 5%-CO_2 for 18 h. Approximately 500 cells were added to IgG-coated wells as a positive control. Following incubation, plates were washed 3 times with PBS-Tween (0.05%), then 3 times with PBS. Goat anti-human IgG-horse-radish peroxidase (Jackson ImmunoResearch; diluted 1/800 in PBS-10% FBS) was added and incubated for 4 h at RT in the dark. Cells were again washed 3 times with PBS-Tween (0.05%), 3 times with PBS, and developed with AEC substrate (BD Biosciences™), according to manufacturer's instructions. ELISPOT plates were then washed with ddH_2O, before analysis using VirusSpot (AID). All assays were performed in triplicate.

2.6.3 Isolation of HBsAg-specific B cells and detection of anti-HBs

In order to isolate HBsAg-specific B cells, ~ 5×10^6 B cells were enriched, as described in section 2.4.1, from $8 \times 10^7 – 1 \times 10^8$ freshly isolated PBMCs (where possible). B cells were incubated with FcR-blocking reagent in PBS supplemented with 10%-FBS and 2mM EDTA, prior to incubation with a combination of mAb (detailed in Table 2.5). Cells were stained for 30 min at 4°C with gentle shaking every 10 min to prevent clumping of cells. Once stained, PBMC were washed twice in ice-cold PBS 10%-FBS with 2 mM EDTA, and re-suspended in a volume of cRPMI corresponding to 5×10^7 cells/ml. Before sorting, cells were filtered through a 70 µm filter-capped polystyrene tubes (BD Biosciences™).

Cells were sorted at low speed on a FACS Aria™ (BD Biosciences™) in either category two or category three conditions (for healthy vaccinated controls or patients with CHB, respectively). Stringent gating was applied to exclude doublet forming cells. Gates defining HBsAg-specific B cells were set using comparison to an FMO (representative gating strategy in Figure 2.2). Sorted cells were collected into polypropylene tubes (BD Biosciences™) containing collection media (cRPMI supplemented with 20%-FBS). Following isolation, cells were washed and cultured as in section 2.6.1.

Table 2-5 Cell surface markers for subset identification in FACS-isolation of HBsAg-specific B cells

Antigen	Fluorochrome	Clone	Manufacturer	Cat No.	Dilution
CD19	APC/Cy7	SJ25C1	BD Biosciences™	557791	2:100
CD3	BV510	OKT3	BioLegend	317332	1:100
AF488-HBsAg-bait		N/A	F. Hoffman-La Roche™	N/A	2:100

For the detection of anti-HBs, HBsAg-specific B cells were sorted and stimulated for 7 d, as above. The number of cells isolated ranged from between 3×10^3 and 1.7×10^4. Culture supernatants (from the second differentiation step) were sent for anti-HBs quantification (Health Services Laboratory, UCLH; Abbott Architect anti-HBs). Samples were also tested in parallel in-house using anti-HBs CLIA kit (AutoBio), according to manufacturer's instructions. CLIA kits were also used for the measurement of serum anti-HBs, where clinical information was unavailable. Serum was diluted 1:100 and tested according to manufacturer's instructions.

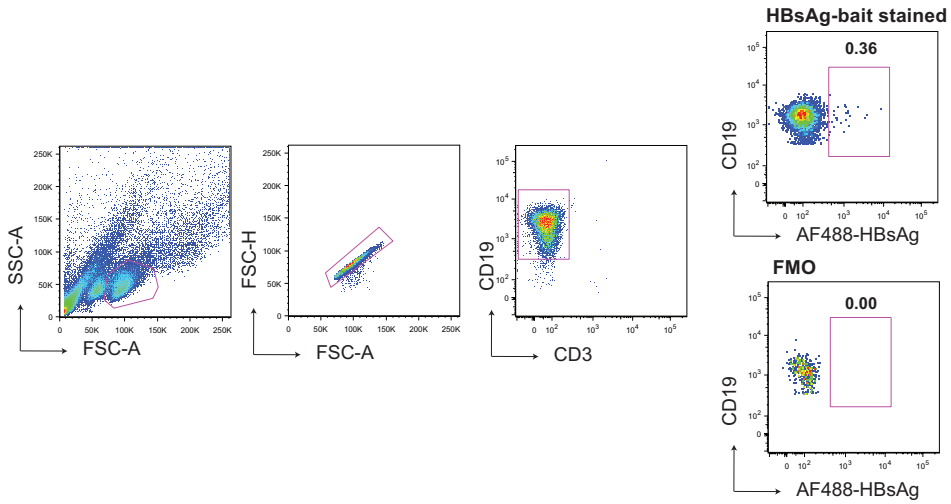

Figure 2-2 Gating strategy for FACS-isolation of HBsAg-specific B cells

Sequential gating strategy used for the isolation of HBsAg-specific B cell by FACs-sorting. B cells were pre-enriched using magnetic separation. Stringent gating was used to isolate viable, single lymphocytes. HBsAg-specific B cells were distinguished through comparison to an FMO (fluorescence-minus-one).

2.6.4 Differentiation of memory B cell subsets into antibody-secreting cells

PBMCs were stained as in section 2.6.3 using mAb detailed in Table 2.6 and sorted into atypical memory and classical memory populations using a FACS Aria™ (BD Biosciences™), as before. Stringent criteria were applied to identify lymphocytes, based on FSC vs SSC, and to remove doublet contamination (Figure 2.3). Between 5×10^4 and 2×10^5 atypical memory B cells were purified using this method; in all cases, a matched number of classical memory B cells were sorted and stimulated as below.

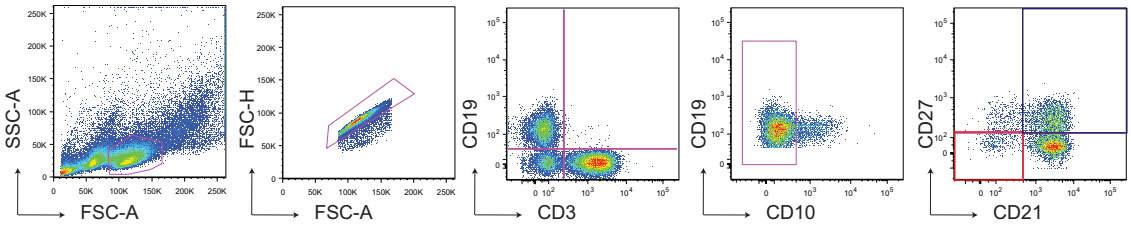

Figure 2-3 Gating strategy for FACS-isolation of memory B cell subsets

Sequential gating strategy used for the isolation of classical ($CD27^+CD21^+$; blue) and atypical ($CD27^-CD21^{low/-}$; red) memory B cell subsets by FACs-sorting. B cells were defined as $CD19^+$ single lymphocytes. Immature B cells were excluded based on CD10 expression.

Sorted cells were first activated using CpG-B ODN 2006 (InvioGen; 500 ng/ml) and cultured as in section 2.6.1. Cells activated with CpG-B but cultured in cRPMI only were used as a control comparison. After culture, cells were stained for plasma cell differentiation using mAb detailed in Table 2.6 and analysed on an LSR-Fortessa-X20™.

For detection of antibody produced during culture, supernatants (from the second differentiation step) were diluted 1:20 and analysed using cytometric bead array for total IgG (BD Biosciences™). Data were collected on a FACS Verse™ (BD Biosciences™). Concentrations of IgG produced were extrapolated through comparison to a standard curve.

CD40-ligand cell line: For some experiments, enriched B cells were cultured in the presence of CD40-ligand expressing Chinese Hamster Ovary cells (CHO) and counterpart CD40-negative cells (obtained from Prof. Claudia Mauri). Prior to culture, cells were deemed mycoplasma negative through commercial testing (GATC Biotech). Cells were cultured in DMEM supplemented with 10%-FBS and 1%-penicillin/streptomycin, and grown to 80% confluency, before irradiation using 50 Gy. 2×10^5 irradiated cells were plated per well and allowed time to settle prior to the addition of B cells.

Table 2-6 Combinations of mAb used for the isolation of memory B cell subsets and the subsequent detection cells with a plasma cell phenotype

Antigen	Fluorochrome	Clone	Manufacturer	Cat No.	Dilution
Pre-sort mAb – for the identification of memory B cell subsets					
CD19	BV786	SJ25C1	BD Biosciences™	563325	2:100
CD3	BV711	OKT3	BioLegend	317328	1:100
CD10	BV605	Ber-ACT8	BioLegend	350218	2:100
CD21	BV421	B-Ly4	BD Biosciences™	562996	2:100
CD27	BUV395	L128	BD Biosciences™	561767	1:100
Post-culture mAb – for the identification of plasma cell differentiation					
CD19	BV786	SJ25C1	BD Biosciences™	563325	2:100
CD20	Alexa-Fluor® 700	2H7	BD Biosciences™	560631	1:100
CD3	BV711	OKT3	BioLegend	317328	1:100
IgD	PE/Cy7	IA6-2	BD Biosciences™	561314	1:100
CD38	PE-dazzle	HIT2	BioLegend	303532	0.5:100
CD138	APC	DL-101	BioLegend	352307	2:100
CD27	BUV395	L128	BD Biosciences™	561767	1:100

2.7 tSNE

tSNE analysis was performed on concatenated flow cytometry data from 10 patients with CHB and 10 healthy controls using default parameters (Iterations, 1,000; perplexity, 20; and θ, 0.5). Analysis was applied to expression data for CD27, CD21, PD-1, FcRL5, CD24, CD38, IgM and IgD for all live $CD45^{+}CD19^{+}CD3^{-}CD20^{-}CD10^{-}$ events.

2.8 Statistical analyses

Statistical analyses were performed in Prism (GraphPad) using the appropriate tests, with significant differences marked on all figures. All test were carried out as two-tail tests. Significance levels were defined as: * = $p<0.05$, ** = $p<0.005$, *** = $p<0.001$, **** = $p<0.0001$.

2.9 Reagents

Table 2-7 Full details, including manufacturers and catalogue number of reagents used through this study

Product	Manufacturer	Catalogue No.
ADAM AccuChip Kit	NanoEnTek	AD4K-200
AEC substrate	BD Biosciences™	551951
AF488-HBsAg-bait	F. Hoffman-La Roche™	N/A
Amicon® Ultra Centrifugal Filters	Merck-Millipore (Sigma-Aldrich®)	UFC905024
anti-HBs CLIA kit	AutoBio	CL0311-2
Brefaldin-A	Sigma-Aldrich®	B6542
Brilliant Stain Buffer	BD Biosciences™	563794
Collagenase IV	Thermo Scientific™	17104-019
Compensation beads	BD Biosciences™	51-90-9001229
CpG-B ODN 2006	InvivoGen	tlrl-2006
Cryovials	Greiner bio-one	123277
Cytofix™	BD Biosciences™	554655
Cytofix/Cytoperm™	BD Biosciences™	554722
DMEM GlutaMAX™-I	Gibco®	61965-026
DMSO	Sigma-Aldrich®	D2650
DNase	Roche	11284932001
EasySep™ Human B cell Enrichment Kit	StemCell™ Technologies	19054
EDTA	Sigma-Aldrich®	E7889

AffiniPure goat anti-human F(ab')$_2$ anti-IgG	Jackson ImmunoResearch	109-006-097
AffiniPure goat anti-human F(ab')$_2$ anti-IgG + -IgM (H+L) + IgA	Jackson ImmunoResearch	109-006-064
F(ab')$_2$ goat anti-human IgM – UNLB	Southern Biotech	2022-01
FcR Blocking Reagent	Miltenyi Biotec®	130-059-901
Ficoll® Paque Plus	GE Healthcare	17-1440-02
Fluo-4AM	Thermo Scientific™	F14201
FoxP3 Buffer Set	BD Biosciences™	560098
Goat anti-human IgG-horse-radish peroxidase	Jackson ImmunoResearch	109-035-088
[Recombinant] HBsAg ayw	F. Hoffman-La Roche™	N/A
HBsAg CLIA kit	AutoBio	CL0310-2
HBSS	Gibco®	24020-091
Heat-inactivated FBS	Thermo Scientific™	10500064
HEPES	Gibco®	15630-056
IFNα	PBL assay sci	11100-1
IL-10	BioLegend	571006
IL-15	R&D Systems	247-ILB-005/CF
IL-2	Peprotech	200-02
IL-21	Gibco®	PHC0214
IL-6 (research grade)	Miltenyi Biotec®	130-093-929
Ionomycin	Sigma-Aldrich®	10634
LEAF™ Purified anti-human PD-1	BioLegend	329911
LEAF™ Purified mouse IgG1κ	BioLegend	401403
LIVE/DEAD™	Invitrogen™	L23105
Mega-CD40-ligand	Enzo	ALX-522-100-C010
MEM essential amino acids	Invitrogen™	11130036
MEM non-essential amino acids	Invitrogen™	11140035
MultiScreen-IP Filter Plate, 0.45 µm	Merck-Millipore (Sigma-Aldrich®)	MAIPS4510
PBS Sterile	Gibco®	14190-094
PBS Tablets	Sigma-Aldrich®	P4417-100TAB
PE-secondary	Thermo Scientific™	A10542
Penicillin/streptomycin	Invitrogen™	15140122
Percoll®	Sigma-Aldrich®	P1644
Phosflow Buffer	BD Biosciences™	558050
phosphorylated BLNK (Tyr96)	Cell Signalling Technology®	#3601S

Probenecid	Thermo Scientific™	P36400
Resiquimod	InvivoGen	tlrl-r848
RPM1 1640	Gibco®	21875-034
Saponin	Sigma-Aldrich®	S-7900
Sodium pyruvate	Invitrogen™	11360039
Total IgG Flex Set CBA	BD Biosciences™	558679
Trypan Blue	Sigma-Aldrich®	31350010
Trypsin-EDTA	Gibco®	25300-062
Tween	Thermo Scientific™	BP337-500
UV (Blue) Live/Dead stain	Life Technologies™	L23105
β-mercaptoethanol	Sigma-Aldrich®	31350010
96-well plate	Sarstedt	83.3925.500

Chapter 3 HBsAg-specific B cells persist in CHB with impaired potential to produce antibody

Chapter summary

Immune control in HBV infection is associated with the presence of functional virus-specific T cells, coinciding with the serological detection of anti-HBs antibodies. However, persistent infections are often distinguished by significant alterations to antigen-specific T and B cell compartments. An important feature of HBV infection is the over-production of HBsAg sub-viral particles: persistently high levels of circulating antigen, along with the tolerogenic environment of the liver, is speculated to promote the functional exhaustion of virus-specific T cell responses in patients with CHB. Taken together, these factors represent a significant challenge to the development and function of HBsAg-specific B cells.

Whilst the quantity and function of HBV-specific T cells has been clearly defined, similarly detailed characterization of HBV-specific B cell responses is lacking. In this chapter, I outline a method to visualise HBsAg-specific B cells directly *ex vivo* in the blood of patients with CHB.

3.1 Introduction

3.1.1 Identification of antigen-specific lymphocytes

A key arm of the adaptive immune system is the instruction of antigen-specific T and B lymphocytes that are able to respond to and generate immunological memory against pathogenic challenge. Central to this ability is a fundamental aspect of the adaptive immune system: antigen specificity. Both T and B lymphocytes – the key effectors of the adaptive immune system - are equipped with receptors that confer their specificity. Similar to B cells, T lymphocytes express a heterodimer consisting of an α and β polypeptide, with structural homology to immunoglobulins. This is referred to collectively as the T cell receptor (TCR). However, unlike B cells that can bind soluble antigen, T cells only recognise peptides presented by MHC. Crucially, both TCRs and BCRs are formed in a process of extensive rearrangement of germline encoded genes, permitting the generation of up to ~10^{10} different specificities.

Quantitative analyses of antigen-specific lymphocytes have provided important insights into the formation of natural immune responses to infection and immunisation. Previous efforts to identify antigen-specific lymphocytes have utilised ELISA-based limiting dilution assays (LDA). These work by measuring the biological activity of specific responder cells on the basis that activity decreases in line with dilution of cells, thereby permitting estimation of the original number of responding cells in culture. This type of assay has the advantage that multiple memory subsets of different antigen specificity can be enumerated from the same sample. When quantifying B cell responses, however, without pre-amplification of antigen-specific populations, cells must be cultured for 2-4 weeks for enough specific antibody to accumulate to a detectable level.

Enzyme-linked immunosorbent spot assays (ELISPOT) circumvent this by quantifying antibody-secreting daughter cells of the memory B cells, rather than accumulated antibody (Slifka and Ahmed, 1996). Accordingly, plasma cells can be detected directly *ex vivo* through ELISPOT, due to their status as a terminally differentiated, antibody-secreting cell. However, this method does not allow for direct characterisation of antigen-specific B cells, nor permit recovery for downstream analyses. Furthermore, both ELISA-LDA and ELISPOT assays rely on the adequate differentiation of memory B cells and assume functional production of antibody. Therefore, they are arguably not representative in settings where there is evidence that antibody secretion or differentiation to antibody-secreting cells (ASCs) may be malfunctioning, as they may underestimate responses through their inability to detect cells that have no proliferative or functional capacity.

In chronic HBV, adaptive immune responses are characteristically weak and transient in nature (Bertoletti and Maini, 2000), thus impeding the description of antigen-specific immune responses. HBV-specific cytotoxic lymphocytes were first demonstrated by chromium release assays following stimulation with synthetic peptides derived from HBcAg and HBeAg *in vitro* (Bertoletti et al., 1991; Penna et al., 1991). These data have since been confirmed by *ex vivo* quantification of these cells

using HLA Class I tetramers (Maini et al., 2000), intracellular cytokine staining (Webster et al., 2004), and ELISPOT analysis post stimulation with either HLA-A2 restricted viral peptides or overlapping pools of peptides spanning the entire HBV genome (Boni et al., 2007). These studies have revealed critical insights into the kinetics of HBV-specific T cells during hepatitis, showing that T cells are profoundly exhausted and depleted in persistent infection, yet are maintained even after clinical recovery in acute hepatitis (Bertoletti and Ferrari, 2016).

Comparatively, very little has been done to study antigen-specific responses against HBsAg; the key viral protein capable of inducing protective antibody responses in HBV (Table 3.1). HBsAg-specific B cells are readily induced by HBsAg-vaccination and can be detected in the periphery and lymphoid tissue, in accordance with detection of protective anti-HBs responses (Barnaba et al., 1985; Böcher et al., 1999; Wang et al., 2015; Ward et al., 2008; Xu et al., 2015). However, frequencies of anti-HBs producing cells are thought to be decreased in the periphery of patients with CHB relative to HBV-vaccinated individuals and compared to patients with acute-resolving infection (Barnaba et al., 1985; Dusheiko et al., 1983; Xu et al., 2015). ELISPOT data enumerating HBsAg-specific IgG-producing cells suggested that the frequency or function of HBsAg-specific B cells may fluctuate according to disease phase, with some patients with active CHB (characterised by raised ALT and viral load) capable of producing anti-HBs to similar levels as HBV-vaccinated or –resolved controls (Xu et al., 2015). However, the majority of these studies are limited by their dependency on antibody production as a readout for HBsAg-specific B cell frequency. Thus, despite these studies, it remains unclear as to whether there is a numerical defect in the formation and persistence of HBsAg-specific B cells, or whether HBsAg-specific B cells persist but are functionally defective. Moreover, co-culture studies using allogeneic T cells from HBV-vaccinated responders and B cells from patients with CHB failed to produce detectable anti-HBs responses, pointing to the existence of B cell specific defects (Barnaba et al., 1985; Dusheiko et al., 1983).

Table 3-1 Summary of key studies investigating HBsAg-specific B cell responses in humans

Study	Methodological approach	Key findings
Xu et al., 2015	HBsAg-specific IgG responses in patients with CHB determined by ELISPOT	- Increased expression of activation makers (CD71 and CD69) on memory B cells in immune active phases, in line with increased production of IgM and IgG - HBsAg-specific IgG responses decreased in patients with HBV relative to HBV-vaccinated healthy controls, but were restored upon resolution of infection
Wang et al., 2015	Measurement of HBsAg-specific memory B cells by ELISPOT in patients with 'anti-HBc alone' HBV	- HBsAg-specific memory B cells were detected in all 'anti-HBc alone' patients and at similar frequencies to patients with resolved infection - Frequency of HBsAg-specific B cells increased following HBsAg-vaccination
Ward et al., 2008	Immunomagnetic separation using fluorescently-conjugated HBsAg in HBV-vaccinated healthy controls	- HBsAg-specific B cells were detectable in PBMCs of HBV-vaccinated healthy controls - Frequency of HBsAg-specific B cells determined by flow cytometry did not correlate with ELISPOT results, or detection of anti-HBs in the serum (by ELISA)
Dusheiko et al., 1983	Detection of anti-HBs by ELISA in the supernatant of B cells stimulated with pokeweed mitogen	- Anti-HBs was undetectable in B cells isolated from chronic HBsAg carriers - Co-culture of T_{FH} cells from HBsAg carriers and B cells from vaccinated healthy controls augmented anti-HBs production
Barnaba et al., 1985	*In vitro* production of anti-HBs following pokeweed mitogen stimulation of PBMCs	- Anti-HBs was detected in supernatants from 24/36 responders to HBV-vaccination, but not from patients with CHB - Co-culture of T helper cells from responders and non-T cells from patients with CHB failed to produce anti-HBs, pointing to a B cell-specific defect
Böcher et al., 1999	Identification of anti-HBs secreting cells by ELISPOT in PBMCs and bone marrow lymphocytes	- HBsAg-specific B cells are compartmentalised within the bone marrow years after HBV-vaccination

To date, there have been no studies that have directly analysed the frequency and phenotype of HBsAg-specific B cells in CHB. Our approach was to use a fluorescently conjugated antigen-bait system, thereby facilitating co-characterisation by monoclonal antibody staining. This system works on the premise that antigen binds specifically to the immunoglobulin of the BCR. Antigen binding can then be detected via coupling to a fluorophore and quantified using flow cytometry (Figure 3.1). Flow-cytometric probes, such as these, were first used in mouse models of infection (Doucett et al., 2005; Hayakawa et al., 1987; McHeyzer-Williams et al., 1993), and have since been used to identify and isolate HIV-specific B cells (Kardava et al., 2014; Knox et al., 2017) and HCV-specific B cells (Boisvert et al., 2016) from human PBMCs, amongst other studies of immunisation. The efficacy of antigen-coupled bait reagent systems to enumerate frequencies of protective HBsAg-specific B cells was first demonstrated in HBV-vaccinated healthy controls. Using a two-step magnetic cell-sorting procedure, HBsAg-specific B cells were enriched from the PBMCs of vaccinated healthy controls at significantly higher frequencies than HBV-naïve controls (Ward et al., 2008). The frequency of HBsAg-specific B cells detected by this method did not correlate with the levels of anti-HBs production quantified by ELISPOT or ELISA, thus demonstrating the ability of this method to detect antigen-specific B cells independent of antibody production.

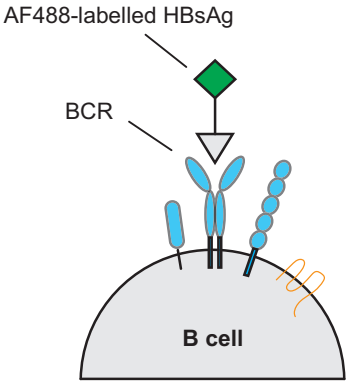

Figure 3-1 Method for detecting antigen-specific B cells using an HBsAg-bait system

Approach used for the identification of HBsAg-specific B cells. HBsAg-specific B cells are detected using flow cytometry via a recombinant HBsAg coupled to an Alexa Fluor-488 fluorophore

3.2 Hypothesis and aims:

In this chapter, I aim to understand how chronic HBV infection may impact the development and function of antigen-specific B cells. It was hypothesised that chronic antigen stimulation, in the form of high levels of circulating HBsAg, may result in HBsAg-specific B cell dysfunction analogous to T cell exhaustion described in CHB. Defects in the frequency or function of HBsAg-specific B cells may contribute to the failure of B cells in patients with CHB to overcome high levels of circulating HBsAg.

The work presented in this chapter will aim to:

1. Test the specificity and sensitivity of a HBsAg-bait system (F. Hoffman-La Roche™) for the identification of HBsAg-specific B cells;
2. Enumerate HBsAg-specific B cells in patients with CHB compared to vaccinated healthy controls and patients with acute-resolving HBV infection;
3. Correlate HBsAg-specific B cell responses with the outcomes of CHB, in particular HBsAg seroconversion and viral control;
4. Assess the ability of HBsAg-specific B cells to produce anti-HBs/form anti-HBs secreting cells.

3.3 Results

3.3.1 HBsAg-bait as a method of detecting HBsAg-specific B cells in PBMCs

To examine whether HBsAg-specific B cells circulate in chronic infection, we first developed and optimised a sensitive method for their direct *ex vivo* detection. Recombinant HBsAg was coupled to AF488-fluorophore (produced in house by F. Hoffman-La Roche™) and used to identify antigen-specific B cells in PBMCs by flow cytometry. The concentration of the HBsAg-bait was carefully titrated to increase the likelihood of detecting true antigen-specific B cells and limit non-specific binding. Rudimentary comparison to a labelled native HBsAg, purified from the serum of active HBV patients, indicated that the frequencies estimated by these two separate methods did not differ significantly (data not shown); for batch consistency, all data was collected using the recombinant antigen.

Stringent gating was used to identify HBsAg-specific B cells. Lymphocytes were delineated on the basis of forward and side scatter, and CD45 expression. Dead cells were excluded from analysis using a fixable live-dead stain. Within the live, lymphocyte population, B cells were identified by CD20 and CD19 expression, with T cells excluded through expression of CD3 (Figure 3.2A). Gates defining HBsAg-specific B cells were set using fluorescence-minus-one staining (FMO; Figure 3.2B) and cross-checked through comparison to $CD19^{-}CD3^{+}$ T cells, which do not express a BCR and therefore should not bind the bait specifically (representative example in Figure 3.2C). HBsAg-specific B cells were calculated as a percentage of $CD45^{+}CD3^{-}CD19^{+}CD20^{+}$ B cells with background fluorescence, determined via corresponding FMO staining, subtracted for each individual. Some non-specific binding was detected in unexposed controls (Figure 3.2B).

This system was validated using a cohort of HBsAg-vaccinated (Engerix B; recombinant HBsAg adsorbed on aluminium hydroxide) and unexposed healthy controls (Figure 3.2D). Only known HBV-vaccine responders were analysed, pre-determined by detectable serum anti-HBs responses. The frequency of circulating HBsAg-specific B cells estimated by this method was in the range of vaccinated donors assessed previously using a 2-step enrichment staining protocol (Ward et al., 2008). A threshold level of detection was set using the mean plus the standard deviation of background staining seen in the unexposed cohort (0.18% of B cells, Figure 3.2D); individuals that had frequencies of HBsAg-bait-binding cells above threshold level were deemed to have a detectable population HBsAg-specific B cells that was absent in unexposed controls. It is noted that this cut-off left 4/24 unexposed donor stains around the limit of detection, indicating that responses around the threshold frequency should be interpreted with some caution.

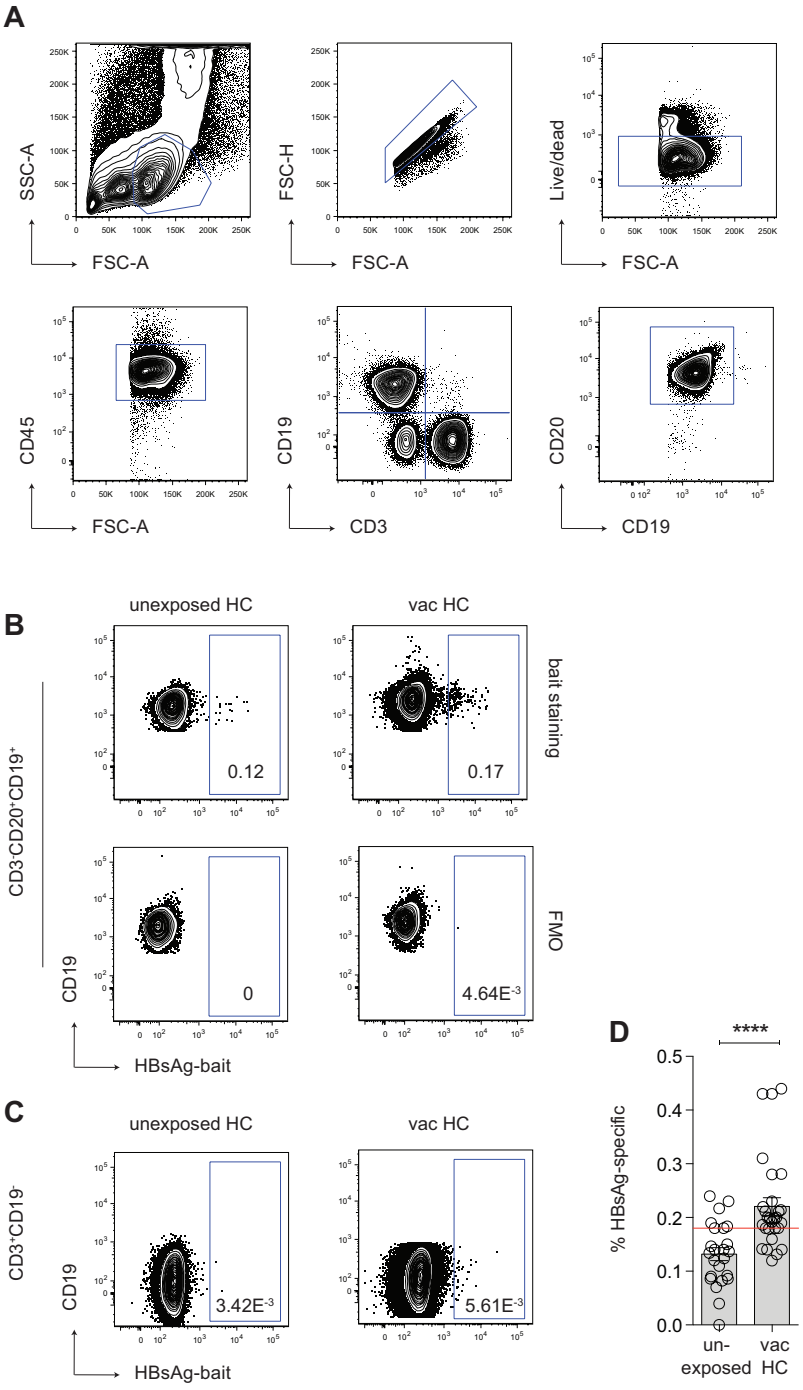

Figure 3-2 Flow cytometric identification of HBsAg-specific B cells

A. Sequential gating strategy for the identification of HBsAg-specific B cells. HBsAg-specific B cells were estimated as a proportion of total B cells, defined as $CD45^+CD19^+CD3^-CD20^+$ **B.** Representative staining of HBsAg-specific B cells in HBV-unexposed and HBV-vaccinated healthy controls (vac HC) identified using an AF488-HBsAg-bait, and compared to FMO (fluorescence-minus-one). **C.** Representative staining using the AF488-HBsAg-bait on $CD3^+CD19^-$ T cells in a vac HC. **D.** Frequency of HBsAg-specific B cells (% of total $CD45^+CD3^-CD19^+CD20^+$) in HBV-unexposed (n=24) and HBV-vaccinated (n=29) healthy controls. Error bars indicate mean ± SEM; ****, $P < 0.0001$; p-values were determined by Mann-Whitney *U* test for unpaired data.

To assess the specificity of this bait system for detecting HBsAg-specific B cells, we used it to track the appearance of protective HBsAg-reactive B cells following HBV vaccination in previously unexposed individuals. Analysis of peripheral B cells, from healthy donors sampled repeatedly during the course of preventative HBV vaccination (Engerix B), revealed that detection of HBsAg-specific B cells, above the background threshold of staining, coincided with the development of a detectable anti-HBs antibody response in sera (Figure 3.3A, B). Two donors who did not complete the course of the vaccination failed to develop a detectable antibody response by ELISA and correspondingly did not develop a population of HBsAg-specific B cells above threshold (Figure 3.3C).

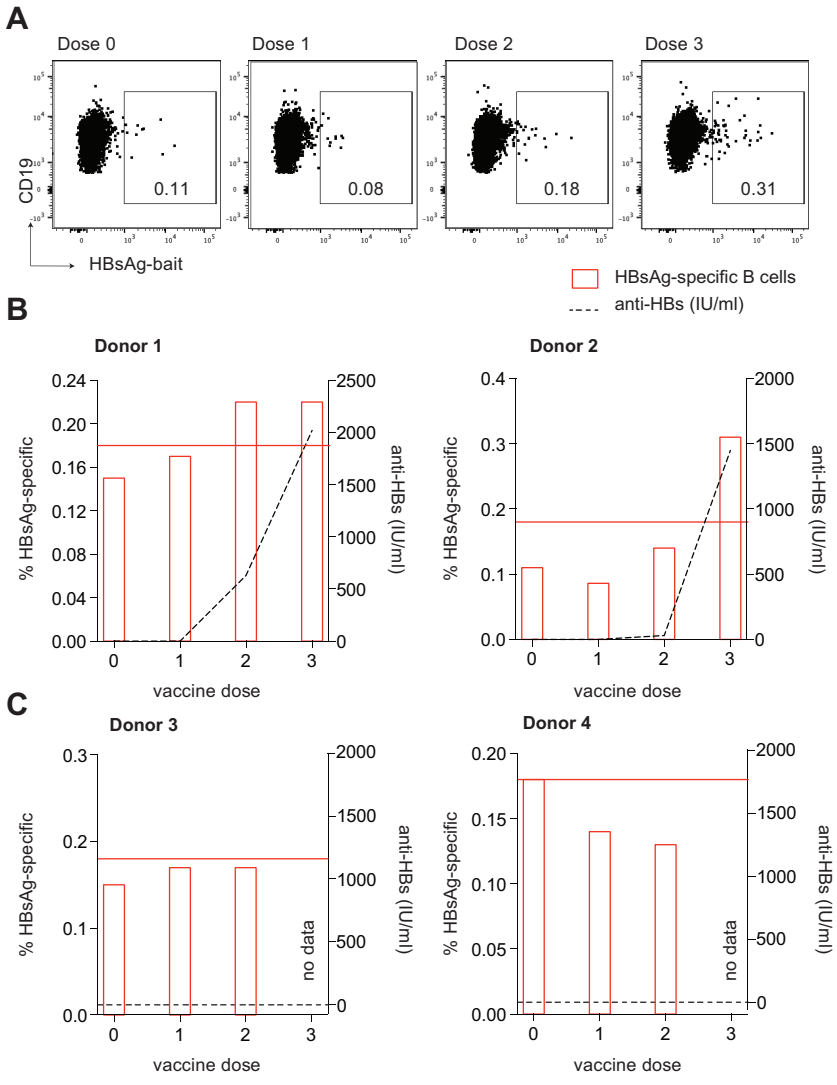

Figure 3-3 Development of HBsAg-specific B cells increase following HBsAg-vaccination

A. Representative staining of HBsAg-specific B cells across the course of HBV-vaccination with Engerix (Donor 2). Samples taken two weeks prior to first dose and seven days post each dose (given 1 and 6 months after the initial dose). **B.** HBsAg-specific B cells (red bars; % of total $CD45^+CD3^-CD19^+CD20^+$) across the course of HBV-vaccination in two healthy donors and **C.** in two healthy donors who did not complete their course of HBV-vaccination. Dashed line represents serum anti-HBs titre (IU/ml) determined by ELISA. Red line delineates threshold level based on mean + s.d of unexposed controls (0.18).

To further validate the sensitivity and specificity of the reagent for the detection of antigen-specific cells, we FACS-sorted the HBsAg-bait-binding and bait-negative B cell fractions from three HBV-vaccinated donors and assessed their functionality after culture using ELISPOT and ELISA. A two-step protocol was employed to promote the activation and differentiation of B cells to plasmablasts and plasma cells (referred to collectively as antibody-secreting cells; ASCs) (Jourdan et al., 2009) (Figure 3.4). anti-HBs produced was measured in the supernatant following ASC differentiation when levels were highest. Cells that bound HBsAg-bait differentiated into HBsAg-specific plasma cells, detectable by ELISPOT, and produced >1000IU/ml of anti-HBs antibody by ELISA (Figure 3.4; UCLH Health Services Laboratory). In contrast, cells from the bait-negative fraction were devoid of HBsAg-reactive responses by ELISPOT and produced no detectable anti-HBs antibody when cultured *in vitro* (Figure 3.4).

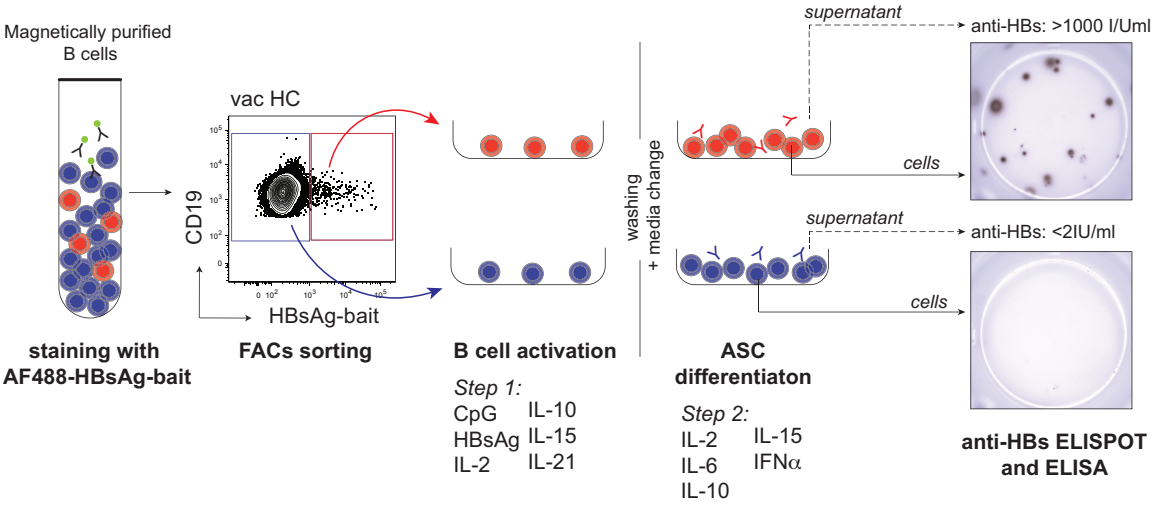

Figure 3-4 *In vitro* differentiation protocol for the differentiation of ASCs

AF488-HBsAg-bait binding and HBsAg-bait deplete cells were FACs-sorted from an HBV-vaccinated control and stimulated polyclonally to activate B cells and promote ASC differentiation. anti-HBs ELISA and anti-HBs ELISPOT assays were performed on culture supernatants and on cultured cells, respectively. Representative of three individual experiments.

3.3.2 HBsAg-specific B cells are detectable in the circulation of patients with CHB

Having validated the specificity of the HBsAg-bait, we then used it to test for circulating HBsAg-specific B cells in a cohort of 84 subjects with CHB. Despite their lack of detectable serum anti-HBs antibodies, we were able to detect HBsAg-specific B cells above the background threshold in 68% of the cohort, at comparable frequencies to a cohort who had been previously vaccinated with HBsAg (representative example and summary data; Figure 3.5A). Both subjects with CHB and vaccinees had significantly higher frequencies of HBsAg-bait-staining B cells than unexposed controls or patients infected with HCV (Figure 3.5A).

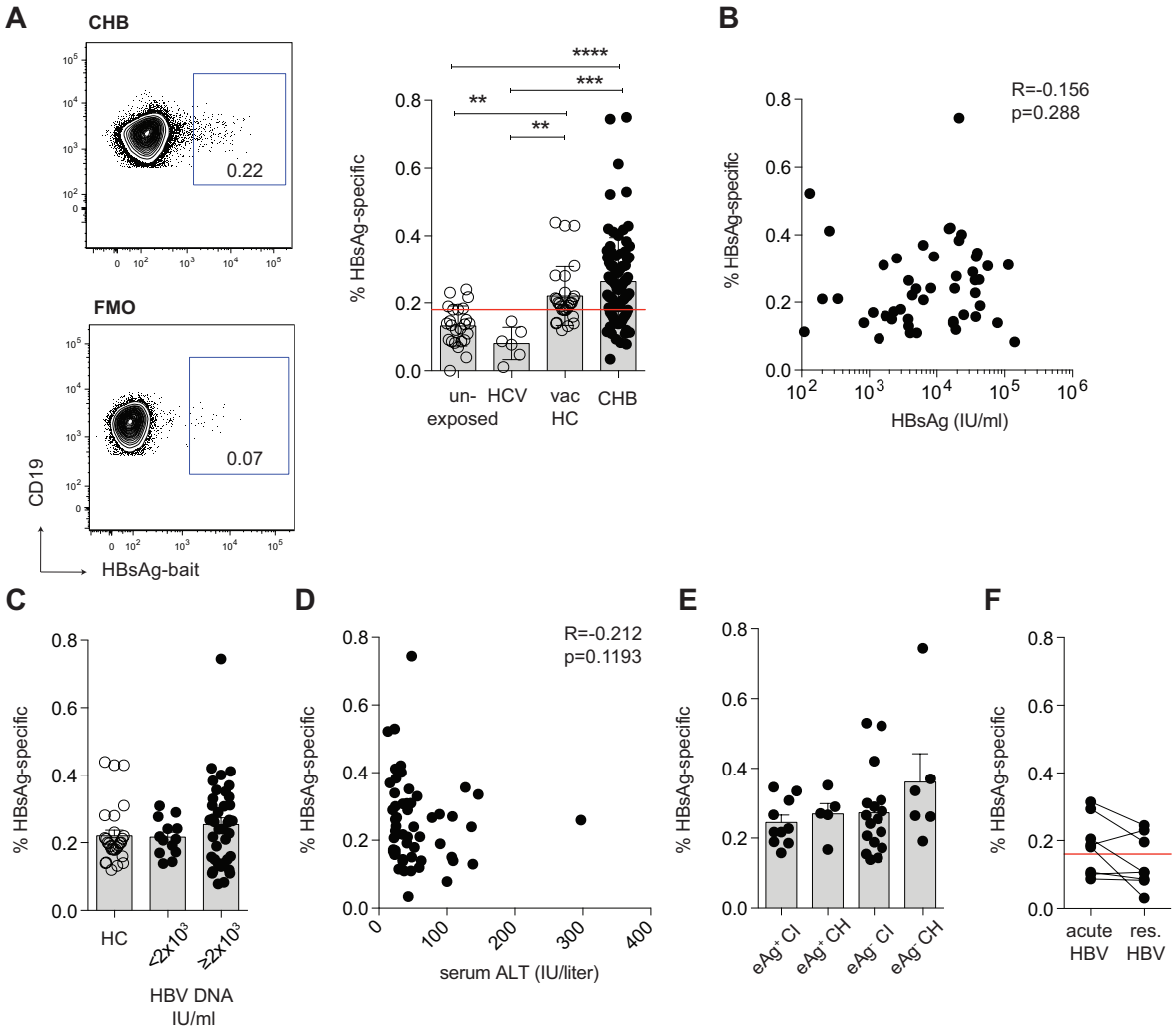

Figure 3-5 HBsAg-specific B cells persist in the circulation of patients with CHB

A. Representative staining of HBsAg-specific B cells in a patient with CHB, compared to FMO (fluorescence-minus-one). Summary plot shows frequency of HBsAg-specific B cells in unexposed healthy controls (n=24), HBV-negative HCV$^+$ patients (n=6), HBV-vaccinated healthy controls (vac HC; n=29) and patients with chronic HBV (CHB; n=84), identified by AF488-HBsAg-bait staining. Frequencies are presented minus paired FMO. **B.** Frequency of HBsAg-specific B cells plotted against serum HBsAg levels (IU/ml; *n*=48). **C.** Summary plot of the frequencies of HBsAg-specific B cells stratified by HBV viral load: n=29 vac HC; n=13 with HBV DNA <2x10^3 IU/ml; and n=39 with HBV ≥ 2x10^3 IU/ml. **D.** Correlative analysis of the frequency of HBsAg-specific B cells and serum ALT (IU/l; n=84). **E.** Summary plot of the frequencies of HBsAg-specific B cells stratified by CHB disease phase: n=10 'HBeAg$^+$ chronic infection' (eAg$^+$ CI, HBeAg$^+$, HBV viral load > 10^7 IU/ml, serum ALT < 40 IU/litre); n=5 'HBeAg$^+$ chronic hepatitis' (eAg$^+$ CH, HBeAg$^+$, HBV viral load > 5 x 10^5 IU/ml, serum ALT > 60 IU/litre); n=17 'HBeAg$^-$ chronic infection' (eAg$^-$ CI; HBeAg$^-$, HBV viral load < 2000IU/ml, serum ALT < 40 IU/litre); and n=6 'HBeAg$^-$ chronic hepatitis' (eAg$^-$ CH, HBeAg-, HBV viral load > 5 x 10^5 IU/ml, serum ALT > 60IU/litre). **F.** Cross-sectional analysis showing the frequency of HBsAg-specific B cells at HBV-acute and -resolved time points (n=8). Red line delineates threshold level based on mean+s.d of unexposed controls (0.18). Error bars indicate mean ± SEM; **, P < 0.01; ***, P < 0.001; ****, P < 0.0001; p-values determined by Kruskal-Wallis test with a Dunn's post hoc test for pairwise multiple comparisons (a, c and e); Spearman's Rank correlation (b and d); and Wilcoxon signed-rank test (f).

To explore the association between HBsAg-specific B cells and disease activity, we next sought to analyse their frequency according to pre-defined clinical parameters. The clinical and virological parameters considered included: serum alanine transaminase (ALT) levels, used as an indicator of liver inflammation; levels of circulating HBsAg; and HBV viral load. Repeated clinical assessment also facilitated comparison of HBsAg-specific B cells according to clinical disease phase, as defined by clinical guidelines (European Association for the Study of Liver; EASL) (Table 1.1).

These were defined as follows:

- $HBeAg^+$ chronic infection: HBV DNA $>10^7$ IU/ml, ALT <40IU/L
- $HBeAg^+$ chronic hepatitis: HBV DNA >5 $x10^7$ IU/ml, ALT >60IU/L
- $HBeAg^-$ chronic infection: HBV DNA <2000IU/ml, ALT <40IU/L
- $HBeAg^-$ chronic hepatitis: HBV DNA >$5x10^5$IU/ml, ALT >60IU/L

The frequency of HBsAg-specific B cells showed no clear relationship with serum HBsAg concentration, HBV DNA, or serum ALT levels, suggesting that there is no direct correlation between the frequency of circulating HBsAg-specific B cells and the levels of viral proteins and/or inflammation (Figure 3.5B-D). Moreover, there were no differences in the frequency of HBsAg-specific B cells according to clinical disease phase (Figure 3.5E), in contrast to previous reports demonstrating an increased frequency of anti-HBs producing cells in immune active phases of disease (Xu et al., 2015).

3.3.3 HBsAg-specific B cells in acute-resolving HBV infection

We next investigated the frequency of HBsAg-specific B cells in patients who control infection and produce protective anti-HBs, by comparing HBsAg-specific B cells in valuable samples of patients sampled during acute and resolved HBV infection. Patients were identified as having resolved infection when HBV DNA and HBsAg were undetectable and ALT levels were normalised (for the purpose of this thesis, ALT levels were considered normal when below 40IU/ml). Paired analysis of HBsAg-specific B cells at HBV-acute versus -resolved time points suggested that HBsAg-specific B cells circulated at similar frequencies in acute compared to chronic infection, and decreased marginally when patients were resampled around the time of HBsAg clearance, although this was not statistically significant (Figure 3.5F).

By obtaining pre-existing clinical information from additional time points (where PBMCs were unavailable), we were able to assess the frequency of HBsAg-specific B cells according to the progression of acute-resolving infection. In doing so, we could examine how the frequency of HBsAg-specific B cells changes as patients develop immunity to HBsAg. Of note, patients with acute HBV infection often present late in clinic, at the onset of clinical symptoms and post the

initial peak in HBV viral load and serum ALT levels. As a result, acute-resolving patients are often sampled towards the resolution of disease, and rarely capture patients in the pre-clinical phase of acute HBV. HBsAg-specific B cells showed a tendency to decrease towards the resolution of disease, although this was not consistent in all patients sampled (Figure 3.6). Where samples were captured earlier in infection, the frequency of HBsAg-specific B cells showed a more marked decrease following the peak of viraemia and serum ALT levels (patients 01-03; Figure 3.6). Combined, these data suggested that the HBsAg-specific B cell compartment displays a tendency to contract following resolution of infection and clearance of HBsAg.

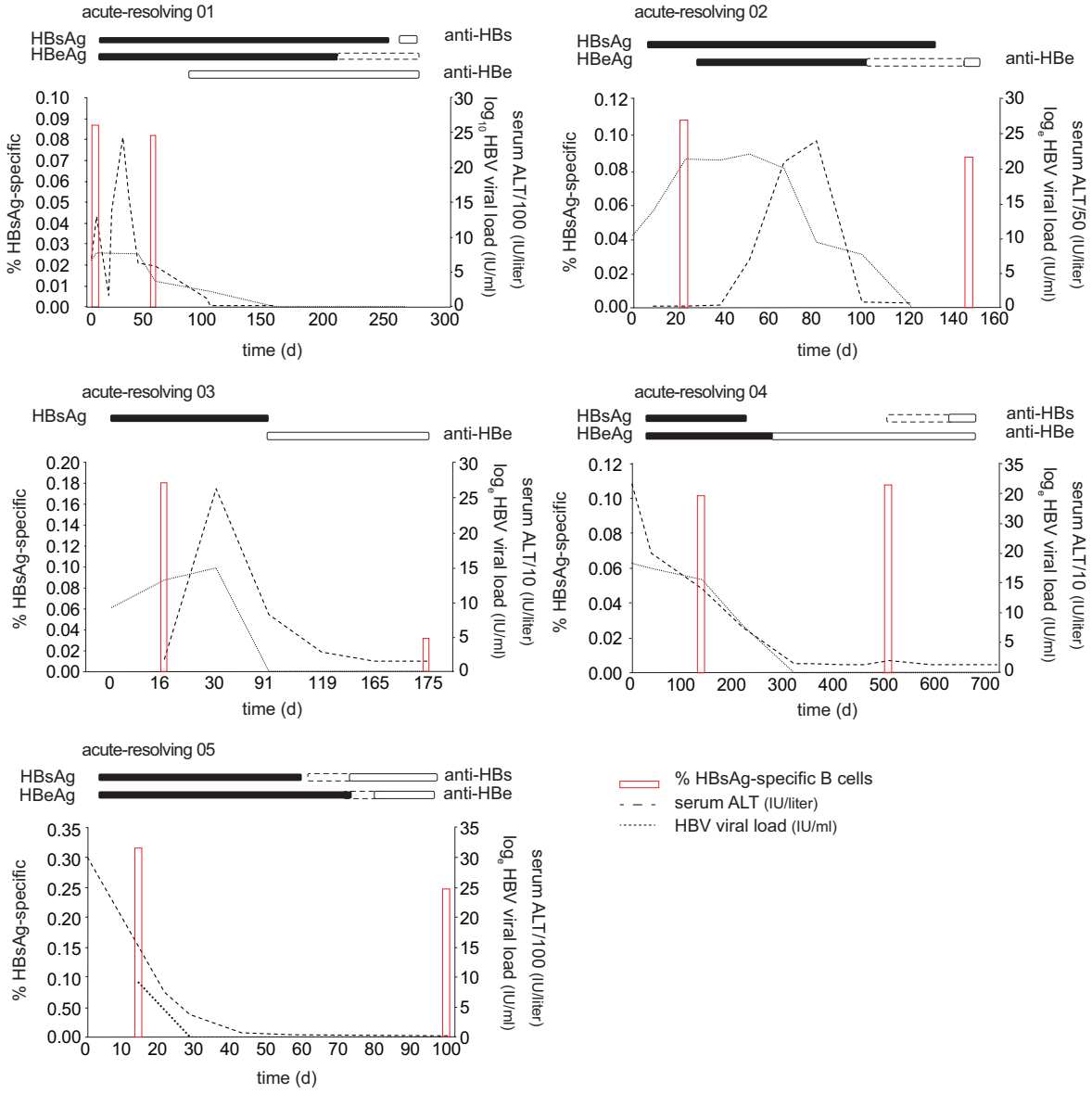

Figure 3-6 Cross-sectional analysis of HBsAg-specific B cells in HBV-acute versus HBV-resolving infection

Clinical data from longitudinal sampling of 5 acute-resolving patients, showing viral load (dotted line; IU/ml), serum ALT (dashed line; IU/litre), and serological data, indicated by the bars (solid bars delineate presence of HBV-viral antigen;

clear bars delineate presence of HBV-viral IgG; dashed bars used where data is not known). Frequency of HBsAg-specific B cells (% of $CD20^+CD19^+$) measured at cross-sectional time points during infection is indicated by the red bars.

Longitudinal analysis of precious samples, taken throughout the time course of acute-resolving infection, permitted more detailed analysis of HBsAg-specific B cell kinetics according to changes in disease parameters. Temporal batch analysis throughout the course of acute-resolving HBV showed no consistent relationship with viral load, serology or liver inflammation (Figure 3.7A).

Finally, analysis of a rare patient with CHB experiencing a flare in disease activity, allowed us to assess the effects of fluctuating levels of viral load and inflammation on HBsAg-specific B cells within the context of CHB. The frequency of HBsAg-specific B cells did not change considerably, despite substantial increases in HBV DNA and ALT (Figure 3.7B). Combined, these data suggest that differences in the clinical manifestation of CHB, and the level of viral replication/control, does not significantly impact the frequency of HBsAg-specific B cells that can be detected in the periphery.

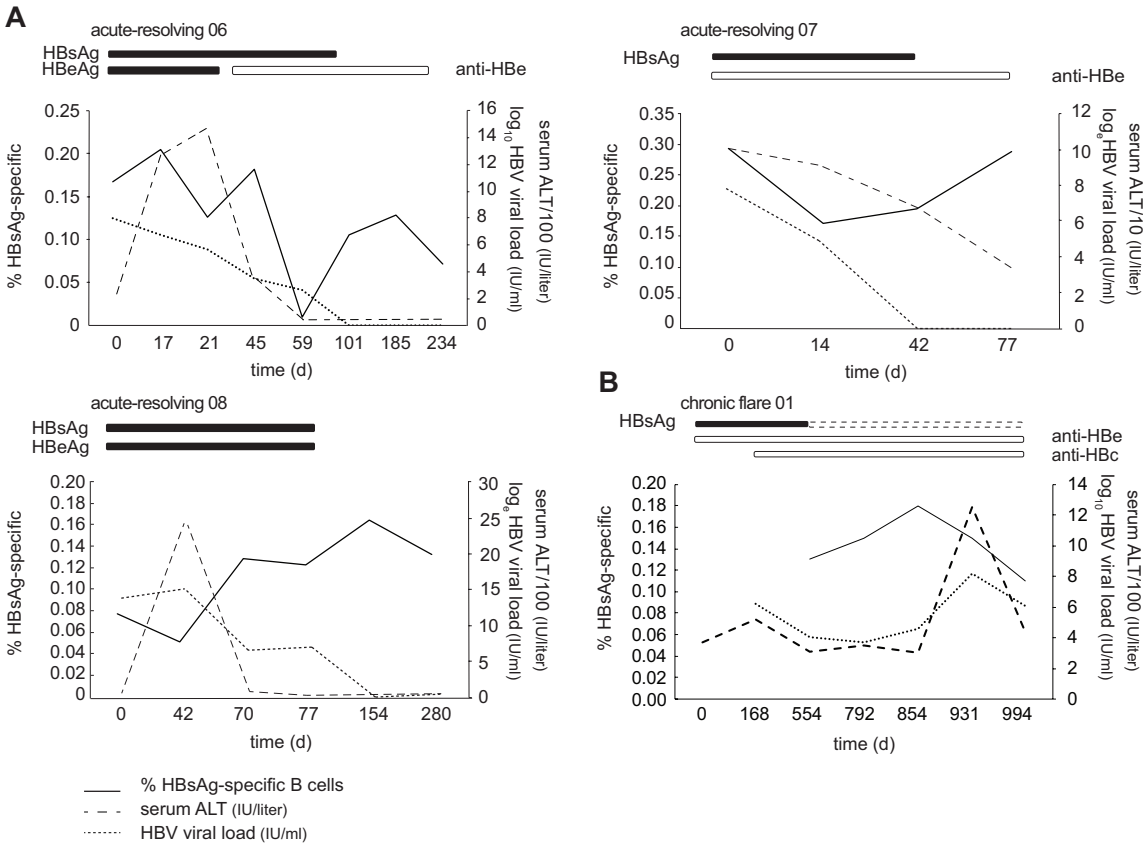

Figure 3-7 Temporal analysis of HBsAg-specific B cells in HBV-acute-resolving infection

Longitudinal analysis of HBsAg-specific B cells (% of $CD20^+CD19^+$) during **A.** acute-resolving infection and **B.** a patient with CHB undergoing hepatic flare. Percentage of HBsAg-specific B cells (black line) are plotted in relation to viral load (dotted line; IU/ml), serum ALT (dashed line; IU/litre) and serological status, as indicated by the bars.

3.3.4 B cells in patients with CHB show defective anti-HBs production *in vitro*

Our results thus far indicated that HBsAg-specific B cells persist in the circulation of patients with CHB at comparable levels to those with acute or resolved HBV infection, and their frequency is not significantly altered by changes in viral load, inflammation or levels of circulating antigen. In light of this, it appears that the apparent lack of anti-HBs in the sera of patients with CHB is not due to a reduced numbers of HBsAg-specific B cells, but instead may arise due to their defective production of anti-HBs. Accordingly, we next investigated whether the HBsAg-specific B cells detected in CHB were capable of differentiating into anti-HBs-producing ASCs.

B cells were magnetically separated from the blood of HBV-unexposed and HBV-vaccinated healthy controls, and patients with CHB, yielding B cells of ~98% purity (Figure 2.1). Cells were cultured using the two-step differentiation protocol utilised in Figure 3.4. Briefly, purified B cells were first activated using polyclonal stimulus (CpG-B ODN 2006) ± antigen-specific stimulus (soluble HBsAg). CpG was used due to its relevance in HBV-physiology and the identification of CpG motifs in HBV DNA (Zhong et al., 2015), alongside its efficacy in promoting plasmablast differentiation and antigen-specific IgG responses (Jahnmatz et al., 2013; Jourdan et al., 2009; Walsh et al., 2013).

Activated cells were expanded using a mixture of B cell homeostatic cytokines, including IL-2, IL-10, IL-15, IL-21, over four days. Following activation, cells were switched to culture medium containing IL-2, IL-10, IL-15, IL-6 and IFNα to promote their differentiation in ASCs and cultured for a further three days. HBsAg was removed from culture through washing. Following differentiation, the number of viable cells was assessed and anti-HBs responses determined by ELISPOT. Analogous wells of stimulated B cells were simultaneously tested for global IgG production to ensure that viable B cells were seeded. This combinatorial approach of B cell activation and cytokine stimulation was required for adequate differentiation to anti-HBs secreting ASCs (Figure 3.8A). HBsAg-reactive responses were detected as large, diffuse spots that approximated responses seen in total-IgG producing controls and were not observed when using an HIV antigen in HIV seronegative individuals (representative example in Figure 3.8B).

Analysis of HBsAg-specific plasma cell differentiation by ELISPOT suggested that global B cells isolated from patients with CHB had defective differentiation compared to healthy vaccinated controls (Figure 3.8B-D). 14/17 patients with CHB had fewer than 10 anti-HBs spot forming cells, compared to 1/7 vaccinated healthy controls (Figure 3.8D). The mean number of spot forming cells of each group was tested for significance using Kruskal-Wallis non-parametric ANOVA. B cells isolated from vaccinated healthy controls were significantly more able to form anti-HBs responses compared to unexposed healthy controls (***) and patients with CHB (*). Conversely, anti-HBs responses were undetectable in unexposed controls (n=5) and were not significantly different to patients with CHB (Figure 3.8D).

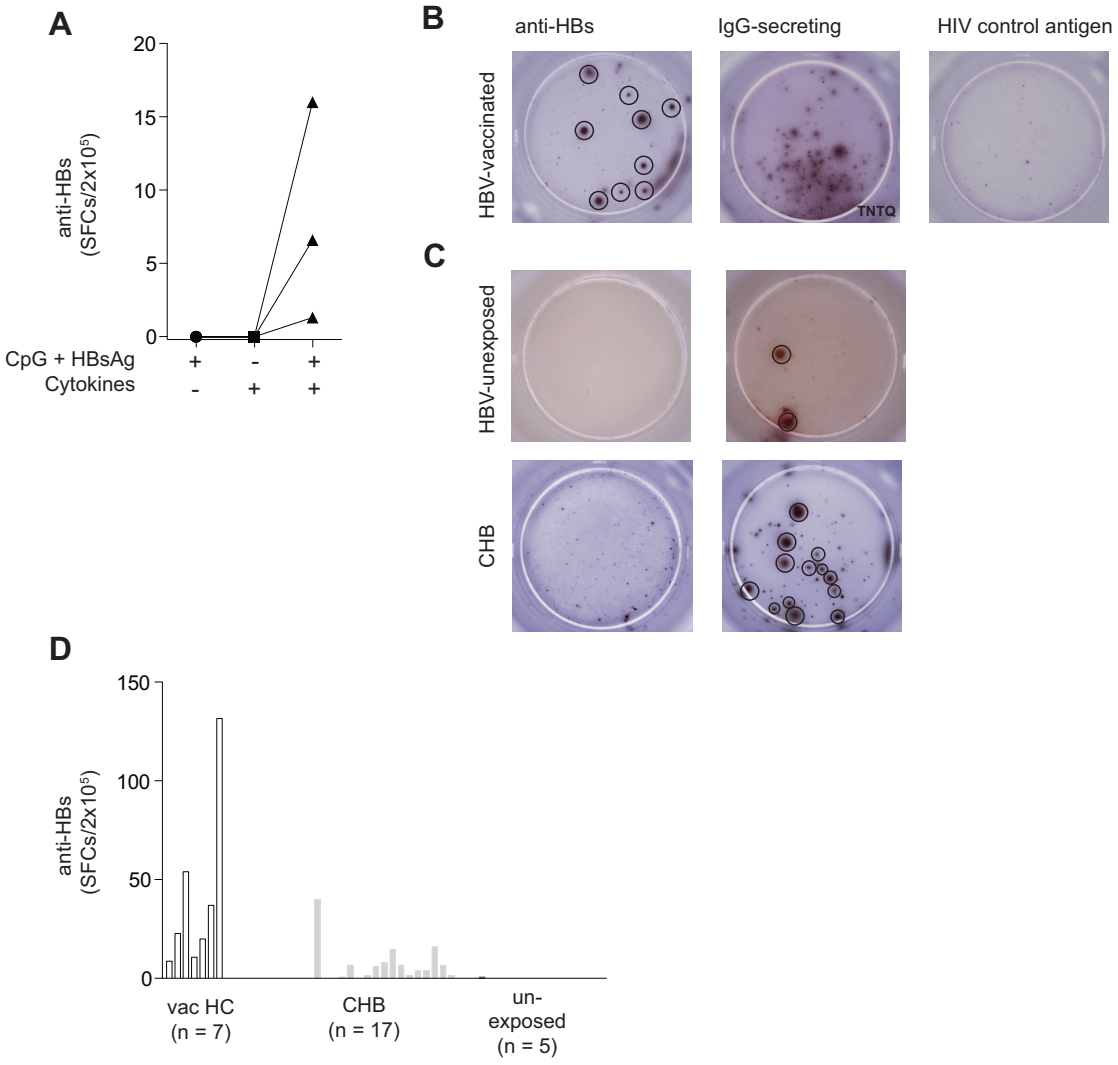

Figure 3-8 Frequency of anti-HBs-producing cells in vaccinated healthy controls and patients with CHB

A. Polyclonal activation of B cells CpG and HBsAg was required in combination with cytokine stimulation to promote differentiation to anti-HBs secreting cells, as shown by ELISPOT in 3 HBV-vaccinated healthy controls. **B**. Representative ELISPOT-well image showing anti-HBs secreting B cells in HBV-vaccinated healthy controls, compared to total-IgG production and control well image using an HIV antigen. Large, diffuse spots were counted (circled). **C.** Representative ELISPOT-well image of anti-HBs secreting B cells in HBV-unexposed controls and patients with CHB, and compared to total IgG production. **D**. Summary plot: anti-HBs-secreting B cells in unexposed controls (n=5), vaccinated healthy controls (vac HC; n=7) and patients with CHB (CHB; n=14), determined by ELISPOT. SFC; Spot Forming Cells. TNTQ; too numerous to quantify. All ELISPOTS were performed using magnetically purified global B cells. Each assay was performed in triplicate and normalised according to the number of cells seeded.

We next directly analysed the anti-HBs producing capacity of HBsAg-specific B cells from patients with CHB. HBsAg-bait-binding B cells that were FACS-sorted from donors with CHB, and cultured as above, failed to produce detectable levels of anti-HBs IgG (<10IU/ml; determined by clinical-grade ELISA, Health Services Laboratory). In contrast, comparable numbers of bait-sorted HBsAg-specific B cells from vaccinated healthy controls differentiated *in vitro* to produce robust levels of anti-HBs (>1000IU/ml) (Table 3.2). The viability of cells post culture was decreased in

patients with CHB relative to healthy controls; however, viable cells were present in both sample types (representative example in Figure 3.9). Direct comparison between cells stimulated with and without HBsAg suggested that residual antigen that may be present in the culture did not affect the detection of anti-HBs, as neither condition showed detectable levels of anti-HBs production. Thus HBsAg-specific B cells were detectable directly *ex vivo* in patients with CHB but showed reduced capacity to produce anti-HBs antibodies upon *in vitro* differentiation, relative to vaccinated healthy controls and consistent with undetectable antibody production characteristic of this stage of infection.

Table 3-2 anti-HBs production of FACs-sorted HBsAg-specific B cells upon *in vitro* stimulation

anti-HBs (IU/ml) in supernatants from stimulated FACS sorted HBsAg-specific B cells (n=3 vac HC; n=4 patients with CHB). Table shows the number of cells seeded and the level of anti-HBs detected (determined by ELISA; performed by Health Services Laboratory, UCLH).

	Identifier	No. of cells stimulated/well	anti-HBs (IU/ml)
HBV-vaccinated healthy controls	HC 01	8834	>1000
	HC 02	5600	>1000
	HC 03	~1.2x10^4	>1000
Patients with CHB	CHB 01	5520	<2
	CHB 02	~1.16x10^4	<2
	CHB 03	9906	<2
	CHB 04	1.7x10^4	<2

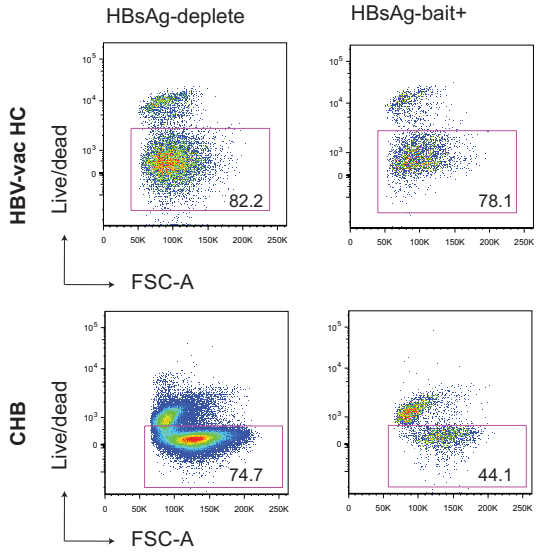

Figure 3-9 Viability of HBsAg-specific B cells post-culture

Representative staining: viability was assessed in FACS-sorted HBsAg-bait binding fractions and HBsAg-deplete fractions following 7-day culture using fixable live/dead staining.

3.4 Discussion

Key elements in the control and elimination of HBV infection include a broad and robust antigen-specific T cell response, as well as the development of neutralizing anti-HBs antibodies leading to HBsAg clearance. Clearance of HBsAg, accompanied by anti-HBs seroconversion, is considered the hallmark of functional cure in HBV; accordingly, chronic hepatitis B infection is characterised by a lack of detectable serum anti-HBs. However, it was not yet known whether this was due to a failure in the development or persistence of HBsAg-specific B cells, or whether dysfunctional HBsAg-specific B cells produce insufficient anti-HBs antibody to overcome the high HBsAg load in patients with CHB.

Our study, in collaboration with a back-to back publication from the group of Antonio Bertoletti (Salimzadeh et al., 2018), is the first to definitively show that HBsAg-specific B cells can persist in the circulation of patients with CHB, yet exhibit defective production of anti-HBs antibodies. This study builds on previous reports of HBsAg-specific B cells in patients with CHB by using a HBsAg-bait system to directly identify HBsAg-specific B cell *ex vivo*. This method has the significant advantage that it bypasses the requirement of antibody production by B cells as a readout for the quantitation of HBsAg-specific B cell numbers, and allows phenotypic characterisation of persisting responses. Thus, unlike previous studies that suggested that HBsAg-specific B cells are depleted (demonstrated via anti-HBs ELISPOT) (Xu et al., 2015), we show that antigen-specific B cells circulate in patients with CHB at comparable frequencies to patients with acute or resolved HBV, and HBV-vaccinated healthy controls. We also present the first direct evidence to show that HBsAg-specific B cells in patients with persistent infection have defective production of anti-HBs, relative to vaccinated healthy controls.

The causes of this functional defect are not yet clear. *In vivo*, HBsAg-specific B cells likely interact directly with circulating HBsAg through their B cell receptor. Prolonged interactions in the instance of chronic infection may result in an overstimulation of the B cell and induce terminal differentiation and exhaustion, analogous to that described in HBV-specific T cells (Bertoletti and Ferrari, 2016). Previous studies have demonstrated functional hyper-activation of global (i.e. non-antigen-specific) B cells in CHB, characterised by elevated expression of CD69 and CD71, that could be exacerbated by CD40-ligand and IFNα stimulation (Xu et al., 2015). Therefore, in the following chapter, I examine the phenotype of HBsAg-specific B cells, compared to functional HBsAg-specific B cells from vaccinated healthy controls, to understand how chronic B cell activation might impair antibody secretion.

Based on our evidence and others, it appears that anti-HBs production is widely heterogeneous between patients with CHB. *In vitro* analysis of anti-HBs production by FACS-sorted HBsAg-bait-binding B cells suggested that antigen-specific B cells in four patients with CHB produce negligible amounts of anti-HBs, in agreement with findings from Salimzadeh et al (Salimzadeh et al., 2018)

and the lack of detectable anti-HBs in patient serum. It is possible that low levels of anti-HBs production by these cells could have been sequestered by residual HBsAg added during B cell activation, thereby leading to an underestimation of the levels of antibody produced. However, direct comparison to cells activated in the absence of HBsAg suggested that this was not the case, and HBsAg-specific B cells isolated from vaccinated healthy controls and stimulated in the same way, still produced robust levels of anti-HBs, in line with the detection of anti-HBs in the serum of HBV-vaccinated individuals.

Building on this, analysis of HBsAg-specific frequencies by ELISPOT also indicated that differentiation to anti-HBs secreting cells is impaired in patients with CHB, relative to vaccinated healthy controls. However, B cells from a small subset of patients possessed anti-HBs producing capability at similar levels to vaccinated healthy controls, in line with previous studies that identified B cells with the potential to form anti-HBs secreting cells (Barnaba et al., 1985; Böcher et al., 1996; Dusheiko et al., 1983). These findings are consistent with the clinical observation that some patients have low anti-HBs serum titre. Low-level production of anti-HBs would also fit with the identification of HBsAg:anti-HBs immune complexes, postulated to exist in patients with CHB (Gerlich, 2007; Madalinski et al., 1991; Madaliński and Bragiel, 1979; Rath and Devey, 1988). Thus, the availability of robust assays for quantitating and/or dissociating circulating HBsAg/anti-HBs complexes would permit a better assessment of the influence of antigen load on HBsAg-specific B cell frequencies and antibody detection.

Our data did not identify a correlation between HBsAg-specific B cells and HBsAg quantified in the serum, nor with the level of HBV DNA, suggesting that there is no direct effect of antigen load on the persistence of HBsAg-specific B cells in the circulation of HBV-infected patients. However, levels of HBsAg in the circulation are not necessarily representative of levels within the liver, and can fluctuate significantly throughout the course of infection. Therefore, correlations between HBsAg-specific frequency and HBsAg load at a single time point is perhaps too simplistic. Although some studies have shown that B cell responses are enhanced (both HBsAg-specific cells and anti-HBs production) in patients in 'immune active' phases of disease association (Vanwolleghem et al., 2015; Xu et al., 2015), our data and others showed no consensus between HBsAg-specific B cell responses and disease phase (Salimzadeh et al., 2018). Instead, our findings point to an overarching impact of HBV infection on the co-ordination of the humoral immune response, for example in the interactions between T helper cells and B cells. This finding perhaps has important implications for the efficacy of therapeutic approaches aiming to rescue the adaptive immune response through the reduction of circulating HBsAg, as it suggests that the reducing antigenemia may help to rescue humoral immune responses.

Circulating antigen-specific B cell frequencies are also unlikely to be representative of memory B cells compartmentalised in lymphoid tissues, particularly after resolution of infection when humoral

immune responses contract. This concept might explain the trend towards fewer HBsAg-specific B cells observed in PBMCs of patients with resolved HBV infection. Follow-up studies, in patients who have long-term resolved infection (i.e. up to 20-30 years post viral clearance), may reveal more striking kinetics of HBsAg-specific B cell responses. In addition, investigation of secondary lymphoid organs could provide important insights into the apparent defects in humoral immunity in CHB. For instance, a diminished propensity to secrete antibody could be indicative of a lack of appropriate CD4 T cell help and/or germinal centre formation. Differences in the formation of germinal centre responses, either in secondary lymphoid organs or ectopically in the liver, may also have relevance in explaining the heterogeneity in anti-HBs production between patients with CHB. Therefore, it will be imperative to sample these sites, where available, and phenotype lymphocytes present. This concept will be discussed in more detail in Chapter Five.

The results of this chapter describe a robust method for the detection of HBsAg-specific B cells in PBMCs of HBV-infected patients. The use of HBsAg-baits for direct *ex vivo* quantification and phenotyping will facilitate further studies of B cell immunity in CHB, but has a number of caveats. Similar to MHC-peptide multimer detection of HBV-specific T cells (Maini et al., 1999), HBsAg-specific B cells are often low frequency and can in some instances approximate levels observed in uninfected controls. Due to the secretion of specific anti-HBs by HBsAg-bait sorted B cells in vaccinated healthy controls, we are confident of the capacity of our bait reagent to detect HBsAg-specific B cells. However, it is likely that non-specific background staining has contaminated our HBsAg-specific population with false positive cells, thus leading to an over-estimation of the levels of HBsAg-specific B cells in our patient cohorts.

Use of a dual-staining antigen-bait can reduce non-specific staining by using two fluorescent parameters to detect a single epitope (Townsend et al., 2001). Non-specific staining from within the normal distribution of the negative population is therefore split between the detection parameters. Accordingly, background staining from each reagent pertains directly to a single fluorescent parameter, reducing the probability of identifying non-specific staining with both reagents (Amanna and Slifka, 2006; Townsend et al., 2001). An alternative approach would be to use a secondary probe with different antigen-specificity to detect background, non-specific staining. Of note, data acquired using a dual-specific bait system, showed comparable frequencies of HBsAg-specific B cells in acute, resolved and chronic stages of infection, once adjusted for background staining (Salimzadeh et al., 2018).

It is also conceivable that HBsAg-specific B cells may be further under-estimated if endogenous HBsAg bound to their BCR *in vivo* prevents bait staining *in vitro*, or if the antibody-binding site on HBsAg in the bait differs from that produced *in vivo*. Antibodies recognise not only linear but also conformational epitopes. Therefore, the recombinant antigen used in this study may have a different conformation to natural envelope protein expressed on the surface of the virion and sub-

viral particles synthesised by HBV-infected hepatocytes. Cells labelled in this way may also include some B cells with low affinity for the antigen. These cells likely would not have the capacity to recognise and generate a response against the antigen *in vivo* (Smith et al., 2017).

With further manipulation of the culture medium it may be possible to enhance the differentiation of B cells from patients with CHB. For instance, studies have indicated that the co-stimulation by CD40-ligand expressing feeder cells can improve differentiation to anti-HBs secreting cells (Salimzadeh et al., 2018). In line with this, a recent report demonstrated the potential importance of the OX40/OX40-ligand axis in maintaining HBV chronicity in a transgenic mouse model of CHB (Publicover et al., 2018). OX40 agonists were shown to successfully promote CD8 and B cell responses, ultimately leading to an increased likelihood of HBV clearance. These data provide important insights into new therapeutic approaches towards functional cure of HBV that should aim to induce co-ordinated T and B cell approaches. In order to identify targets, we must first understand the mechanisms by which this population may be functionally restrained. To do so, it will be critical to ascertain the phenotype of HBsAg-specific B cells, addressed in the next chapter.

Chapter 4 B cells with an atypical memory phenotype are expanded in CHB

Chapter overview

It is increasingly recognised that chronic infections can be accompanied by substantive changes to the composition of the memory B cell compartment. These alterations have been postulated to result in significant impairments in the generation of humoral immunity. In the previous chapter, I showed that HBsAg-specific B cells persist in the circulation of patients with CHB, yet have an impaired ability to differentiate into anti-HBs-producing cells. In this chapter, I address mechanisms that may be responsible for restraining the antiviral potential of antigen-specific B cells, by examining the phenotype and antiviral function of memory B cell subsets within the global and antigen-specific compartments of patients with CHB.

4.1 Introduction

4.1.1 CD27^{-}CD21$^{low/-}$ B cells – atypical memory B cells

Chronic immune stimulation is associated with perturbations of B cell and T cell responses. One such phenomenon is the expansion of CD27^{-}CD21$^{low/-}$ B cells in response to chronic antigen stimulation that have several characteristics of dysfunctional memory B cells. These cells are referred to variously as "exhausted memory B cells" or "tissue-like memory B cells" in HIV and "atypical memory B cells" in malaria; however, cells with a similar phenotype are also found to be expanded in a number of autoimmune conditions and in ageing healthy individuals, although their role is yet to be properly elucidated. For ease, these cells will be referred to as atypical memory B cells (atMBCs) throughout, unless otherwise specified.

First described in the tonsils, CD27^{-}CD21$^{low/-}$ B cells were shown to produce immunoglobulin (IgM/G/A) when stimulated with IL-2, IL-10 and CD40-L (Ehrhardt et al., 2005). These cells displayed morphological similarities to plasma cells, yet were transcriptionally distinct, expressing lower levels of Blimp-1, spliced XBP1 and IRF4 – key transcriptional regulators of plasma cell fate. Despite transcriptionally resembling plasma cells, CD27^{-}CD21$^{low/-}$ B cells displayed many markers characteristic of memory populations, including CD69, CD80 and CD86. However, unlike conventional memory B cells, they lacked expression of CD27, and expressed high levels of the integrin CD11c, alongside a newly characterised inhibitory receptor, Fc receptor-like protein 4 (FcRL4) - shown to bind IgA. *In vitro* studies demonstrated that CD27^{-}CD21$^{low/-}$ were unresponsive to BCR stimulation, yet could proliferate and produce immunoglobulin in response to cytokines and CD40 stimulation. Follow-up work, using more discriminatory antibodies, has since characterised subsets within the CD27^{-}CD21$^{low/-}$ population that express the closely related receptor, Fc receptor-like protein 5 (FcRL5) (Li and Tolnay, 2017). Due to their location in the tonsils, it was postulated that these cells might be adapted to their local environment; however, the function of CD27^{-}CD21$^{low/-}$ B cells in this setting remains unclear.

Humoral dysfunction in infectious and inflammatory settings has previously been associated with a reduction of CD21 expression on B cells in both human HIV infection (Benedetto et al., 1992; Scott et al., 1993) and in animal models of inflammatory disease (Takahashi et al., 1997; Takamatsu et al., 1999). CD21 (complement receptor 2; Cr2) is expressed on the surface of mature lymphocytes and interacts with CD19 and CD81 to form the B cell co-receptor (Carroll, 1998). Co-ligation of the BCR and co-receptor complex assists in lowering the threshold level of antigenic stimulation required by the cell to activate. This was first demonstrated through experiments using anti-IgM and anti-CD21 monoclonal antibodies whereby co-ligation of CD21 decreased the amount of anti-IgM required by the cell in order to proliferate (Carter and Fearon, 1992). In line with this, cross-linking of CD21 in instances where B cell activation is sub-optimal (i.e. without

BCR stimulation), can assist in B cell activation (Tedder et al., 1994). In particular, the presence of CD21 within the co-receptor complex has been postulated to act as an important focal point in the transduction of signals from the innate immune system to regulate humoral immunity. These signals are transduced through the binding of CD21 to its ligand C3d - a component of the complement pathway that binds to the pathogen surface and targets it to B cells and FDCs. Initial evidence for the role of CD21 in regulating humoral immunity came from the finding that $CD21^{-/-}$ mice had significant impairments in antibody production, and both primary and secondary humoral immune responses to T cell dependent antigens (Ahearn et al., 1996). Additional work later suggested that the expression of the CD19/CD21 complex leads to enhanced processing of complement-tagged antigens and augmented presentation to T-helper cells (Cherukuri et al., 2001).

4.1.2 atMBCs in chronic viral infection

Early work suggested that HIV-viraemia was associated with an expansion of a B cell subpopulation that expressed lower levels of CD21. In comparison to $CD21^{high}$ B cells, $CD21^{low}$ B cells exhibited reduced proliferation and increased propensity towards cell death (Moir et al., 2001, 2004). Upon further investigation, it was suggested that among $CD21^{low}$ B cells were two distinct populations of memory B cells – "activated memory B cells" $CD27^{+}CD21^{low/-}$ B cells and "tissue-like memory B cells" $CD27^{-}CD21^{low/-}$ – that were expanded in the peripheral blood of HIV-infected patients compared to healthy controls (Moir et al., 2008). This latter population could be further defined by the expression of FcRL4 - a Fc receptor homologue with the ability to inhibit BCR signalling via recruitment of the Src homology 2 domain-containing phosphatase 1 (SHP-1) (Ehrhardt et al., 2003). These cells also expressed additional B cell inhibitory molecules, including CD22, CD72, LAIR-1 and CD85j (Moir et al., 2008). Akin to inhibitory receptor expression on exhausted T cells in models of chronic infection (Wherry et al., 2007), these cells were suggested to represent an equivalent population of dysfunctional B cells that accrued as a result of chronic antigenic exposure.

Indeed, tissue-like memory B cells were shown to have defective proliferation and decreased immunoglobulin diversification, leading to the production of lower specificity antibodies (Moir et al., 2008). siRNA knock-down of inhibitory molecules on their cell surface led to moderate rescue of BCR-mediated proliferation, and improved differentiation to HIV-specific antibody secreting cells (Kardava et al., 2011). Importantly, cells of this phenotype were shown to be concentrated in HIV-specific B cell populations (Kardava et al., 2014; Knox et al., 2017) pointing to a role in chronic antigen exposure in the development of this dysfunctional population. Comparatively, B cells against short-lived infections such as influenza and tetanus were enriched within classical memory, $CD27^{+}CD21^{+}$ populations (Kardava et al., 2014).

In HIV-infection, activated memory B cells ($CD27^{+}CD21^{low/-}$) also constitute a significant proportion of gp-140-specific B cells (Moir and Fauci, 2013). These cells are generally described as an intermediary population between classical IgG^{+} memory B cells and $CD27^{-}CD21^{low/-}$ B cells (Kardava et al., 2014; Moir et al., 2008). Activated memory B cells express the highest levels of activation markers of all subsets considered, including CD95 and CD80, and demonstrate a predisposition towards apoptosis (Moir and Fauci, 2013). Overall, an expansion of dysregulated antigen-specific cells is postulated to contribute to the inefficiency of the humoral immune response in HIV-infected patients.

4.1.3 atMBCs in malaria

In a manner similar to chronic viral infections, protective immunity to *Plasmodium falciparum (Pf.)* can take years to develop and requires repeated exposures. In the absence of re-exposure, immunoglobulin levels decline and humoral immunity to infection wanes (Kinyanjui et al., 2007). Thus, it is thought that humoral immunity is not efficiently formed, or is functionally impaired in malaria. Evidence for the formation of memory B cells in malaria is conflicting: memory B cells were undetectable despite the presence of antibodies to blood-stage antigens (Dorfman et al., 2005; Wipasa et al., 2010), yet others found that memory B cells were induced incrementally with repeated seasonal exposure (Weiss et al., 2010) and could be long lived (Ndungu et al., 2012). Combined, these data suggest that *Pf.* infection induces memory B cell responses, but is less efficient than other infections or vaccinations that induce long lasting protection.

In accordance with B cell responses in HIV infection, one explanation for this may be antigen-driven expansion of dysregulated atypical memory B cells (atMBC). These cells are significantly enriched in individuals living in malaria-endemic areas and, similar to tissue-like memory B cells, express high levels of inhibitory receptors, including FcyRIIB, CD85j and CD22. Indeed, genome wide expression profiling of B cells in malaria patients revealed a high degree of overlap between genes found to be highly expressed in atMBCs, and tissue-like memory B cells in HIV-infected individuals (Portugal et al., 2015). However, in contrast to tissue-like memory B cells, malaria-induced atMBCs did not express FcRL4, and instead expressed high levels of the closely related molecule FcRL5. atMBCs expressing FcRL5 were significantly more impaired in their ability to secrete antibody, relative to FcRL5-negative counterparts (Sullivan et al., 2015). In line with high FcRL5 expression, atMBCs displayed an impaired ability to respond to BCR cross-linking through reduced phosphorylation of key BCR signalling molecules (Portugal et al., 2015). In contrast, other reports suggest that atMBCs are highly activated and can secrete neutralising antibody (Muellenbeck et al., 2013). Several observations have since linked the expansion of atMBCs to chronic exposure to *Pf.*, showing clear correlations between the frequency of atMBCs and the intensity of parasite

transmission (Ayieko et al., 2013; Illingworth et al., 2013; Muellenbeck et al., 2013; Nogaro et al., 2011; Portugal et al., 2012, 2015; Weiss et al., 2009).

4.1.4 $CD27^{-}CD21^{low/-}$ B cells in ageing and autoimmunity

An expansion of $CD21^{low}$ B cells with many similarities to atMBCs has now been described in numerous models of autoimmunity, where they are thought to directly contribute to the production of autoreactive humoral responses. In contrast to chronic viral infections, these cells are more commonly characterised by expression of the integrin CD11c, in combination with low or negative expression of CD21.

$CD21^{low}CD11c^{+}$ B cells are enriched in the blood of elderly individuals (≥65 years) relative to younger controls (Colonna-Romano et al., 2009; Frasca et al., 2017), and in the spleen of aged mice (Hao et al., 2011; Knode et al., 2017; Ratliff et al., 2013; Rubtsov et al., 2011; Rubtsova et al., 2015). These cells were shown to proliferate robustly in response to TLR stimulation, yet demonstrated diminished responses to BCR ligation. At present, their role in healthy individuals is poorly understood. One possible outcome of their expansion in healthy individuals may be the dysregulation of T_{FH} responses. Whilst it was shown that age-associated B cells were potent antigen-presenting cells (Rubtsov et al., 2015), *in vitro* co-culture of age-associated B cells skewed differentiation of naïve CD4 T cells towards a Th17 phenotype (Hao et al., 2011). Therefore, it is possible that the antigen-presenting capacity of age-associated B cells differs from that of conventional APCs. In addition, age-associated B cells have also been postulated to inhibit the survival of early B cell subsets (pro-B cells), thereby disrupting normal lymphopoesis in aged individuals (Riley et al., 2017).

Several groups have described an expansion of $CD21^{low}CD11c^{+}$ B cells in settings of autoimmunity, including in patients with common variable immune deficiency (CVID) and rheumatoid arthritis (Isnardi et al., 2010; Rakhmanov et al., 2009; Warnatz et al., 2002). These cells expressed a unique array of chemokine receptors, including CXCR3 and CXCR6, supporting their migration towards inflammatory tissue sites, and their accumulation in the bronchoalevolar space and synovial fluid (Rakhmanov et al., 2009). Similar to atMBCs, these cells were hyporesponsive to BCR triggering, demonstrated by decreased calcium flux and proliferation upon stimulation. Importantly, these cells are thought to contribute to the formation of humoral autoimmunity. $CD21^{low}$ populations in CVID are enriched for autoreactive BCRs capable of recognising nuclear and cytoplasmic structures (Isnardi et al., 2010); likewise, in murine models of lupus-like disease, depletion of $CD11c^{+}$ age-associated B cells reduced the amount of autoantibodies detected in the serum (Rubtsov et al., 2011).

CD10-CD27-CD21$^{low/-}$ B cell populations similar to atMBCs, that fail to respond to either BCR or TLR stimulation, are also expanded in settings of HCV-associated mixed cryoglobulemia (Charles et al., 2008; Visentini et al., 2012). Similar cells were also enriched in HCV-associated cirrhosis, where they were again hypo-proliferative in response to BCR stimulation, but retained a similar capacity for antibody production relative to conventional memory B cells (Doi et al., 2014).

The commonalities of CD27-CD21$^{low/-}$ B cells in chronic infection and autoimmunity are summarised in Figure 4.1.

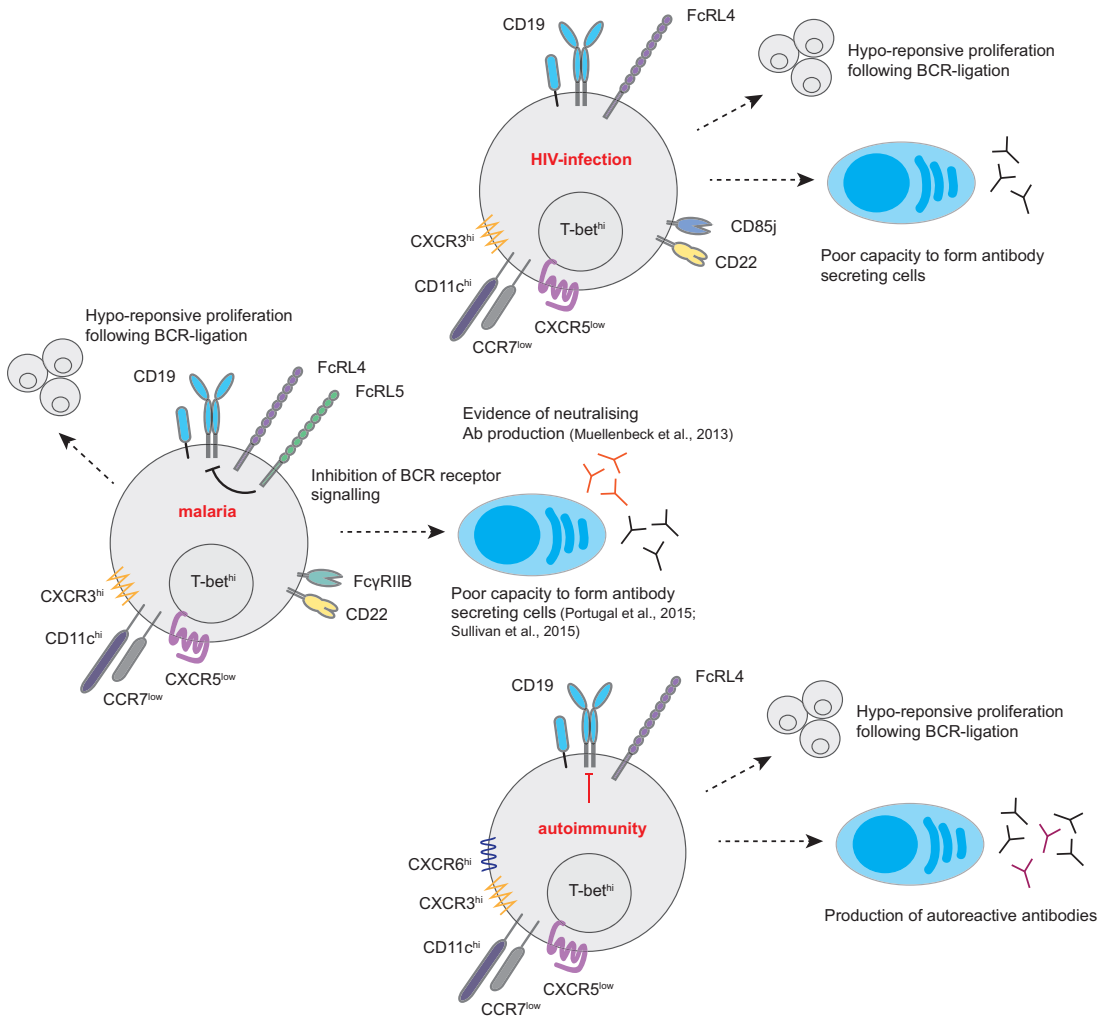

Figure 4-1 Summary of CD27^{-}CD21$^{low/-}$ B cells described in HIV infection, malaria and autoimmunity

CD27-CD21$^{low/-}$ B cells are expanded in settings of HIV-infection, malaria and autoimmunity where they demonstrate hypo-responsiveness to BCR stimulation and altered expression of lymphoid homing receptors. In particular, CD27-CD21$^{low/-}$ B cells in chronic infection can be characterised by upregulation expression of inhibitory receptors.

4.1.5 T-bet expression in B cells

Common to atMBCs, tissue-like memory B cells and age-associated B cells is the expression of molecules not generally expressed by classical memory B cells, including CD11c and the transcription factor T-box expressed in T cells (T-bet) (Myles et al., 2017). T-bet was first identified as an essential regulator of naïve CD4 T cell differentiation within the Th1 lineage. Since then, T-bet has also been shown to be influential in regulating the development and effector functions of NK cells, CD8 T cells, dendritic cells, monocytes and now B cells (Kallies and Good-Jacobson, 2017; Simonetta et al., 2016; Zhang et al., 2018). Co-ordinated expression of T-bet throughout the immune system is thought to promote Th1 responses; for instance, T-bet expression has been shown to drive cytolytic responses in CD8 T cells, whilst simultaneously repressing Th2 responses (Oestreich and Weinmann, 2012). One of the first observations of T-bet in murine B cells showed that expression promoted production of IFNγ, where B cells had been stimulated through CD40-ligation and in the presence of Th1 cytokines (Szabo et al., 2000). IFNγ production by B cells was in turn associated with promotion of CD4 T cell differentiation, and accordingly Th1 polarisation of B cell responses. To date, numerous studies have shown T-bet expression on memory B cells in inflammatory and infectious disease settings (reviewed in Karnell et al., 2017).

T-bet^{+} B cells have recently been shown to expand in both HCV and HIV infection (Knox et al., 2017), with commonalities in cell surface marker expression linking this increased expression of T-bet to the previously described expansion of CD27^{-}CD21$^{low/-}$ B cells. In HIV, peripheral T-bet^{hi} B cells demonstrate decreased expression of CD21, concurrent with inhibitory receptor expression and in line with descriptions of dysfunctional tissue-like memory B cells (Knox et al., 2017; Moir et al., 2004, 2008). Moreover, T-bet^{+} memory B cells expressed elevated levels of CXCR3, in accordance with its elevated expression on atMBCs (Moir et al., 2008; Weiss et al., 2009) and other CD21^{low} B cell subsets (Rakhmanov et al., 2009).

T-bet expression in B cells isolated from patients with HCV has been directly linked to persistent antigenemia. T-bet^{+}CD27^{-}CD21$^{low/-}$ B cells were significantly increased in the periphery of patients with chronic HCV infection, relative to healthy controls (Chang et al., 2017). Maintenance of T-bet expression was dependent on sustained viraemia, whereby effective antiviral treatment reduced the number of T-bet^{+} atMBCs. *In vitro* experiments demonstrated that exposure of B cells to HCV-viraemic plasma led to an expansion of T-bet^{+} B cells with a CD27^{-}CD21$^{low/-}$ phenotype. In contrast, exposure of B cells from HCV-resolved individuals to pre-treatment serum re-induced T-bet expression (Chang et al., 2017).

A recent study shed light on the mechanisms by which viral infection may promote T-bet expression, demonstrating that engagement of the BCR, TLR-7 (capable of detecting viral single-stranded RNA (Sepehri et al., 2016)) and IFNγ receptors in B cells played a synergistic role in driving the expression of T-bet in B cells (Rubtsova et al., 2013). In line with this, malaria-induced

IFNγ stimulation similarly induced the expansion of T-bet^{hi} atMBCs, whereby exposure of naïve B cells to the supernatants of malaria-exposed PBMCs led to the induction of T-bet^{hi} cells with commonalities to atMBCs, that could be abrogated via IFNγ blockade (Obeng-Adjei et al., 2017). Thus, viral infection can be a significant driver in the expression of T-bet in memory B cells. In the absence of IFNγ, T-bet expression can be induced in TLR-activated B cells via a complex reciprocal relationship of T_{FH}-associated cytokines, namely IL-4 and IL-21 (Naradikian et al., 2016a). IL-21 has since been shown to drive age-associated B cells in autoimmune mice via regulation of the transcription factor, interferon-regulatory factor-5 (IRF5) (Manni et al., 2018).

The common expression of inhibitory and migratory markers, coupled with decreased expression of CD21, on atMBCs and T-bet^{hi} memory B cells in chronic infection, make it tempting to suggest that a T-bet driven transcriptional program may regulate the formation of atypical memory. However, how these cells contribute to viral disease remains unclear and appears to contradict the description of atMBCs as a dysfunctional cell population. Recent studies in murine LCMV infection have implied that T-bet^{+} B cells may be critical to control of persistent viral infections. Specifically, T-bet regulates immunoglobulin class-switching to IgG2a/c in mice (Peng et al., 2002; Rubtsova et al., 2013) – the key antibody isotype involved in murine Th1 responses. Furthermore, sustained T-bet expression was required by IgG2a^{+} memory B cells for their survival and functionality (Wang et al., 2012). As IgG2a (equivalent of IgG1 in humans) is the most efficient inducer of antibody-mediated cellular cytotoxicity (Kipps et al., 1985), T-bet expression in B cells was postulated to drive viral clearance. Thus, mice that have lost T-bet expression in B cells develop persistent viral infection (Barnett et al., 2016). In humans, T-bet^{+} B cells positively correlated with the levels of antiviral IgG1 and IgG3 in patients with HIV infection, and were found to dominate the antigen-specific response f. Thus, in the same way it promotes Th1 responses in T cells, T-bet can also be said to directly regulate antiviral responses by B cells.

4.2 Hypothesis and aims

In this chapter, I assess the impact of HBV infection on global and antigen-specific B cells. As reviewed, chronic antigen stimulation has been postulated to drive the expansion of dysfunctional memory B cell subsets. As such, it was hypothesised that persistent stimulation of antigen-specific B cells by circulating HBsAg may lead to perturbations in the composition of HBsAg-specific memory B cells. An expansion of dysfunctional cells within this population may contribute to defects in humoral immunity, as observed in patients with CHB.

The work presented in this chapter will aim to:

1. Dissect the composition of HBsAg-specific memory B cells according to well-described memory B cell populations in vaccinated healthy controls and patients with acute-resolving and chronic HBV infection;
2. Compare the phenotype of HBsAg-specific B cells in HBV-vaccinated healthy controls to patients with CHB;
3. Analyse the antiviral function of memory B cell subsets with regard to BCR signalling, cytokine production and differentiation to antibody secreting cells;
4. Identify putative targets for the rescue of HBsAg-specific responses in patients with CHB.

4.3 Results

4.3.1 B cells with an atypical phenotype are enriched in the HBsAg-specific compartment in patients with chronic infection

To investigate the noted defect in antibody production by HBsAg-specific cells circulating in CHB (Section 3.3.4), we first sought to dissect their composition according to well-described memory B cell subsets discussed above. To do this, we took advantage of the capacity to stain HBsAg-specific B cells directly *ex vivo*, thereby allowing their characterisation by surface phenotype.

Memory B cell subsets were defined using a multi-parametric sequential gating strategy, shown in Figure 4.2A. B cells were identified according to CD19 expression compared against CD3. By gating on $CD19^{+}CD3^{-}$ lymphocytes, we excluded $CD19^{-}CD3^{-}$ NK cells and $CD19^{-}CD3^{+}$ T cells from analysis. Expression of CD10 and CD20 was used to exclude immature transitional B cells and plasma cells/blasts respectively, on the basis that they may otherwise represent a proportion of $CD21^{low/-}$ B cells. Within the $CD10^{-}$ mature B cell population, memory B cell subsets were defined as follows: classical memory B cells (cMBC) $CD27^{+}CD21^{+}$; activated memory B cells (actMBC) $CD27^{+}CD21^{-}$; and atypical memory B cells (atMBC) $CD27^{-}CD21^{low/-}$. These gates were set through comparison to fluorescence minus one controls (FMO) (example in Figure 4.2B) and/or cross-referenced to non-B cell populations of $CD21^{low/-}$ lymphocytes (namely T and NK cells) and CD27 expression on total lymphocytes (expressed divergently on B, NK and T cells) (example in Figure 4.2C). Mature naïve B cells, defined as $CD27^{-}CD21^{+}CD10^{-}$ B cells, were also excluded.

Alternative flow cytometric approaches have also been described that distinguish naïve B cells from memory B cell subsets based on the expression of two developmentally regulated markers, CD38 and CD24. This strategy identified peripheral B cell subsets as follows: $CD19^{+}CD38^{high}CD24^{high}$ as immature and transitional B cells; $CD19^{+}CD38^{int}CD24^{int}$ as mature naïve B cells; and $CD19^{+}CD38^{int/-}CD24^{high}$ as memory B cells (Vossenkämper et al., 2013). Within this latter population, atMBCs have been described to have decreased or negative expression of CD24 (Buffa et al., 2013; Rakhmanov et al., 2009; Thorarinsdottir et al., 2016). Therefore, we cross-referenced our gating strategy to this alternative phenotypic classification. In doing so, we showed that atMBCs have decreased expression of CD38 and CD24 relative to naïve and immature B cells (Figure 4.2D). Expression of CD38 was moderately diminished on atMBCs compared to $CD24^{hi}$ $CD38^{int}$ classical and activated memory B cells, with CD24 expression more significantly reduced, in line with previous reports. Thus, atMBCs are clearly distinct from other circulating populations, including naïve B cells and conventional memory B cell subsets.

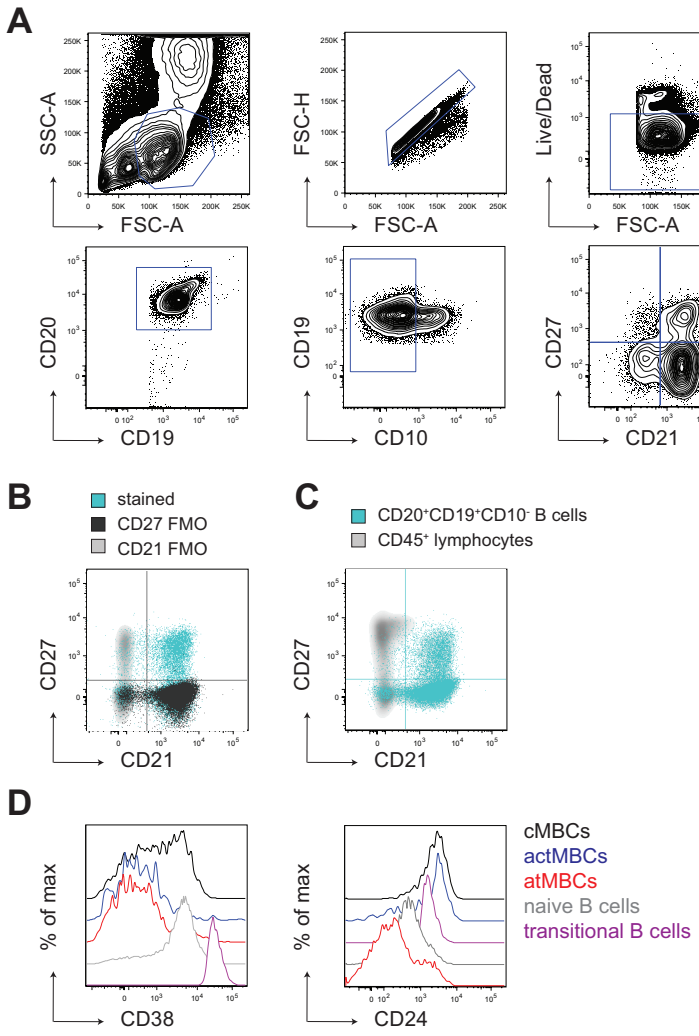

Figure 4-2 Flow cytometric identification of peripheral atMBCs

A. Sequential gating strategy for the identification of memory B cell subsets. Memory B cell subsets were identified on the expression of CD27 and CD21; (atypical 'atMBC' $CD27^{-}CD21^{-}$; activated 'actMBC' $CD27^{+}CD21^{-}$, classical 'cMBC' $CD27^{+}CD21^{+}$; gated on $CD45^{+}CD19^{+}CD3^{-}CD20^{+}CD10^{-}$ lymphocytes). **B.** Representative example of CD27 and CD21 flow cytometric staining on mature B cells (blue; $CD20^{+}CD19^{+}CD10^{-}$) compared to FMOs. **C.** Representative example of CD27 and CD21 staining on mature B cells (blue; $CD20^{+}CD19^{+}CD10^{-}$) compared to $CD45^{+}$ lymphocytes (grey). **D.** Representative example of the expression levels of CD38 and CD24 on memory B cell subsets, compared to naïve and transitional B cells.

Pre-gating on live CD45$^+$CD3$^-$CD20$^+$CD19$^+$CD10$^-$ HBsAg-specific B cells, we compared the contribution of each subset as a proportion of HBsAg-specific memory B cells in healthy vaccinated controls and patients with CHB (Figure 4.3A). Naïve B cells (CD10$^-$CD27$^-$CD21$^+$) were excluded from analysis. Cells with a cMBC phenotype were the principal component of HBsAg-specific B cells in healthy vaccinated controls (Figure 4.3B), consistent with generation of protective antibody responses upon vaccination. However, in patients with CHB, the HBsAg-specific cMBC population was contracted and replaced by B cells with an atMBC phenotype, alongside a smaller expansion of activated memory B cells (Figure 4.3B). atMBCs, and to a lesser extent activated memory B cells, constituted a larger proportion of HBsAg-specific B cells in patients with CHB compared to vaccinated healthy controls; in contrast, cMBCs were decreased as a proportion of HBsAg-specific B cells in patients with CHB relative to healthy vaccinated controls (Figure 4.3C). Given the limited expansion of actMBCs within HBsAg-specific B cells, analysis of HBsAg-specific memory B cells concentrated on comparative differences between cMBCs and atMBCs, henceforth.

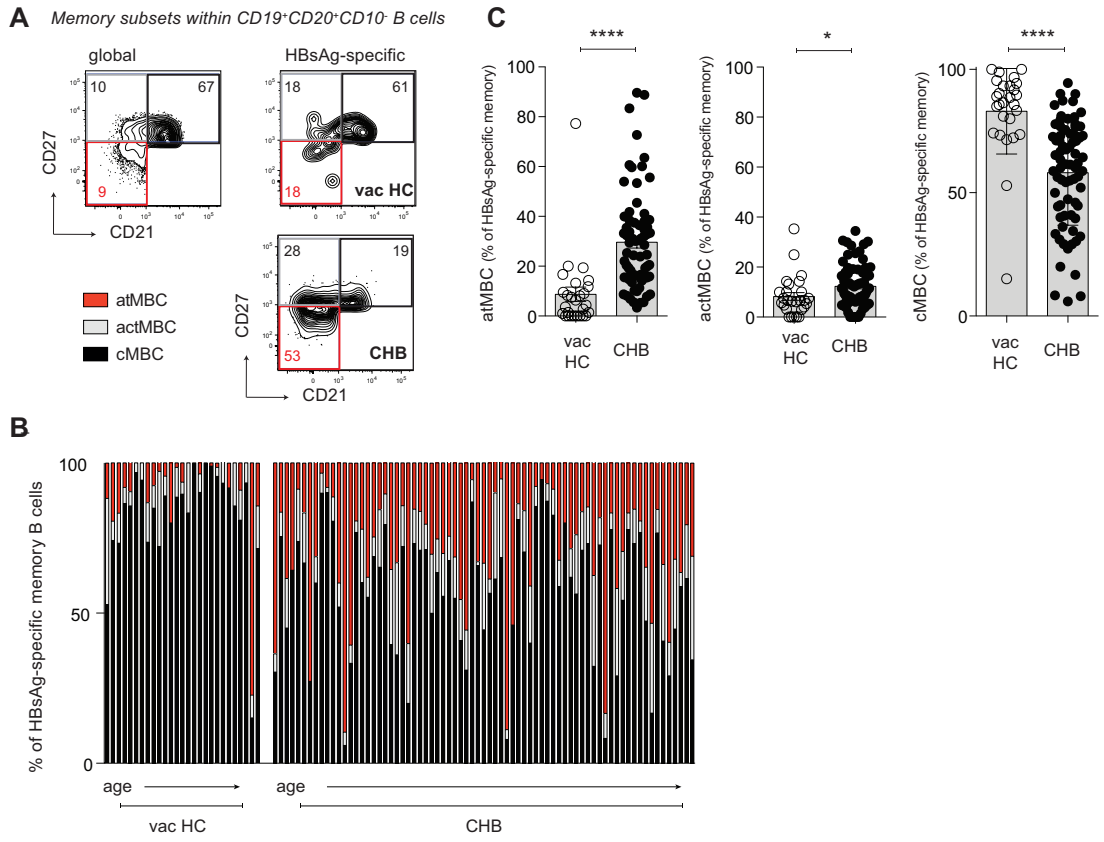

Figure 4-3 atMBCs are enriched within the HBsAg-specific compartment in patients with CHB

A-B. Representative staining and cumulative data: HBsAg-specific memory B cell subsets (atypical 'atMBC' CD27$^-$CD21$^-$; activated 'actMBC' CD27$^+$CD21$^-$, classical 'cMBC' CD27$^+$CD21$^+$; gated on CD45$^+$CD19$^+$CD3$^-$CD20$^+$HBsAg$^+$CD10$^-$) in HBV-vaccinated healthy controls (vac HC; n=27) and patients with chronic HBV (CHB; n=73). Subsets are presented as a proportion of memory subsets (mature B cells (CD10$^-$) minus naïve B cells (CD27$^-$CD21$^+$)). Each bar represents an individual. Individuals are ordered by increasing age (range: vac HC = 21-89yrs; CHB = 23-71yrs). **C.** Summary plots comparing the frequencies of HBsAg-specific memory subsets between vac HC (n=27) and patients with CHB (n=73). Error bars indicate means ± SEM; *, $P < 0.05$; ****, $P < 0.0001$; p-values determined by Wilcoxon signed-rank test (c).

We next examined factors that might be driving this expansion of atMBCs within the antigen-specific compartment by comparing the frequency of HBsAg-specific atMBCs to pre-defined clinical parameters (outlined in Section 3.3.2). As CD27$^-$CD21$^{low/-}$ B cells have previously described to expand with ageing (Colonna-Romano et al., 2009; Frasca et al., 2017), we first ruled out that the observed expansion was not attributable to age. The proportion of atMBCs within HBsAg-specific B cells did not correspond with the age of the individual, as indicated by the order of the bars (Figure 4.3B); thus, the expansion of atMBCs within HBsAg-specific B cells in patients with CHB was not associated with ageing. Likewise, the frequency of HBsAg-specific atMBCs was not affected by the degree of liver inflammation, as indicated by serum ALT level, nor showed any association with clinical phase of disease and viral load, as defined by EASL guidelines (Table 1.1.) (Figure 4.4A-C). However, the frequency of atMBCs as a proportion of HBsAg-specific memory B cells showed a weak inverse correlation with HBsAg load (R=-0.434; Figure 4.4D).

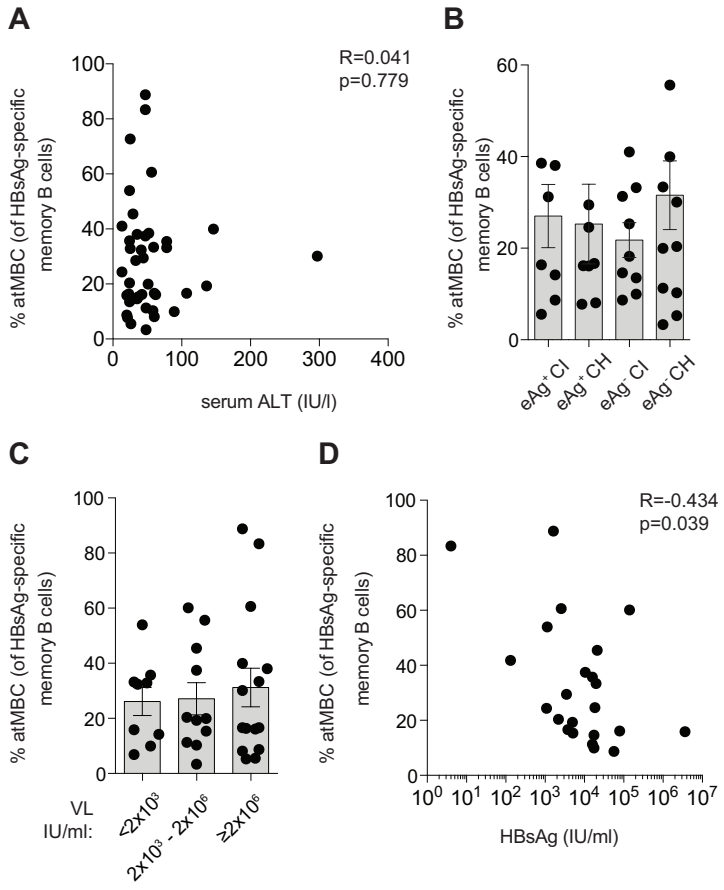

Figure 4-4 Frequency of HBsAg-specific atMBCs according to clinical parameters

A. Frequencies of atMBC as a proportion of HBsAg-specific memory B cells, correlated with levels of serum ALT (n=42) and **B.** stratified by disease phase: n=8 'HBeAg$^+$ chronic infection' (eAg$^+$ CI; HBeAg$^+$, HBV viral load > 10^7 IU/ml, serum ALT < 40 IU/litre); n=5 'HBeAg$^+$ chronic hepatitis' (eAg$^+$ CH, HBeAg$^+$, HBV viral load > 5 x 10^5 IU/ml, serum ALT > 60 IU/litre); n=10 'HBeAg$^-$ chronic infection' (eAg$^-$ CI; HBeAg-, HBV viral load < 2000 IU/ml, serum ALT < 40 IU/litre); and n= 6 'HBeAg$^-$ chronic hepatitis' (eAg$^-$ CH, HBeAg$^-$, HBV viral load > 5 x 10^5 IU/ml, serum ALT > 60 IU/litre). **C.** Frequency of HBsAg-specific atMBC stratified by viral load: n=9 with HBV DNA < 2 x 10^3 IU/ml; n=11 with HBV DNA 2 x 10^3 – 2 x 10^6 IU/ml; and n=15 with HBV ≥ 2 x 10^6 IU/ml; and **D.** correlated with HBsAg (IU/ml;

n=38). Error bars indicate mean ± SEM; p-values were determined by Spearman's Rank correlation (a and d) and Kruskal-Wallis test with a Dunn's post hoc test for pairwise multiple comparisons (b and c); where no p-value is shown, significance was not reached.

This type of analysis only provided a snapshot of the antigen-specific memory compartment at the sampled time point. Thus, to expand on these data, we next examined the kinetics of HBsAg-specific atMBCs in patients undergoing HBsAg-seroconversion during acute-resolving infection, in samples where there were sufficient numbers of HBsAg-specific B cell to reliably dissect antigen-specific memory populations (cut off set at a minimum of 50 antigen-specific cells). Analysis of these samples facilitated temporal assessment of memory B cell subsets within the HBsAg-specific compartment according to dynamic changes in well-defined clinical characteristics within an individual, including viral load, ALT levels and presence/absence of viral proteins, namely HBsAg and HBeAg. The proportion of antigen-specific memory B cells with an atMBC phenotype (shown by the blue bars) decreased in line with falling levels of HBV DNA, and was particularly marked after HBsAg loss (Figure 4.5). Longitudinal sampling in one patient in particular (patient 08) allowed us to uniquely analyse HBsAg-specific atMBCs prior to the ALT flare characteristic of symptomatic acute infection i.e. during the pre-clinical phase of infection when viraemia is typically maximal (Dunn et al., 2009; Webster et al., 2000). These data suggested that HBsAg-specific atMBCs were elevated at the height of disease activity, and decreased following resolution of disease, supporting the role of HBsAg and disease activity in impairing antigen-specific B cell responses.

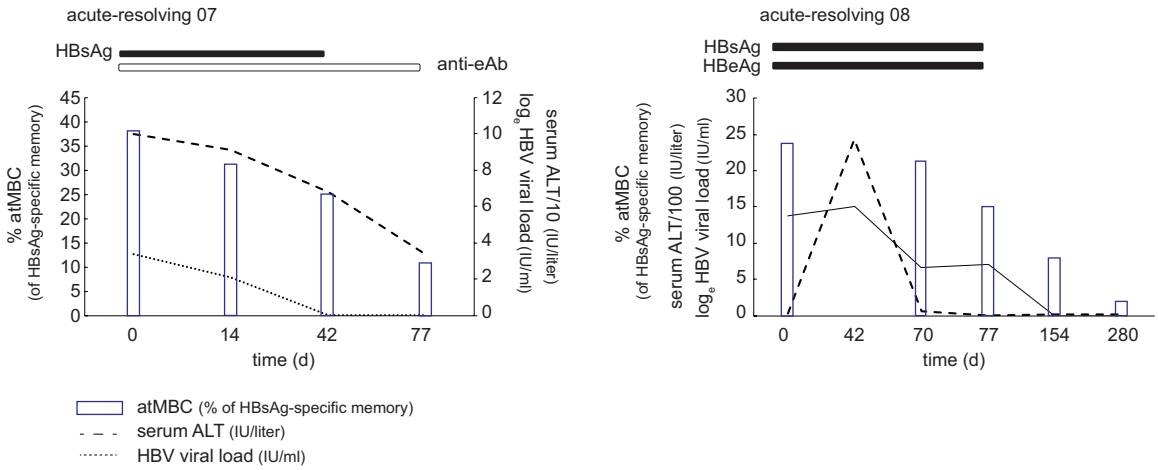

Figure 4-5 Temporal analysis of HBsAg-specific atMBCs throughout the time course of acute-resolving HBV infection

Temporal analysis of the frequency of HBsAg-specific atMBCs throughout acute-resolving HBV-infection. Percentage of HBsAg-specific atMBCs (blue bars) are plotted in relation to viral load (dotted line; IU/ml), serum ALT (dashed line; IU/litre) and serological status as indicated by the bars.

4.3.2 B cells with an atMBC phenotype are expanded in the global B cell compartment

To investigate whether this expansion of atMBCs was exclusive to HBsAg-specific B cells, we next examined the global circulating B cell memory population (i.e. regardless of antigen-specificity). As in Section 4.3.1, memory B cell subsets were defined on the basis of CD27 and CD21 expression and are presented as a proportion of mature CD10$^-$ B cells. Whilst there was no significant difference in the frequency of global cMBCs between patients and healthy controls (Figure 4.6A), there was a significant expansion of atMBCs and activated memory B cells (actMBCs) in the global compartment of patients with CHB (Figure 4.6B). In contrast, naïve B cells were significantly decreased as a proportion of global B cells in patients with CHB (Figure 4.6C). Importantly, atMBCs represented a larger proportion of HBsAg-specific memory population (calculated as a proportion of combined memory B cell subsets) compared to the global B cell compartment, pointing to an enrichment of atMBCs within the antigen-specific pool (Figure 4.6D).

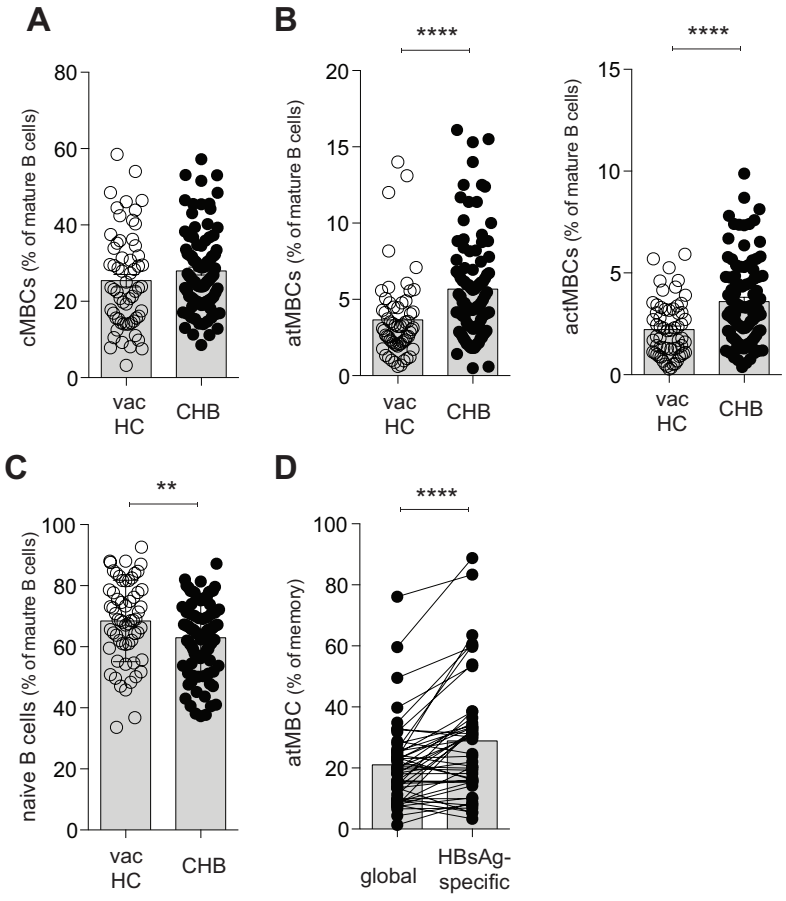

Figure 4-6 atMBCs are expanded in the global B cell compartment in patients with CHB

A-C. Frequency of global memory B cell subsets and **(C)** naïve B cells in patients with CHB (n=96) compared to healthy controls (vac HC; n=61). **D.** Paired analysis of cells with an atMBC phenotype in the global compared to HBsAg-specific compartment (n=49 patients with CHB). Frequency of atMBCs subsets are presented as a proportion of memory subsets (mature B cells (CD10$^-$) minus naïve B cells (CD27$^-$CD21$^+$)). Error bars indicate means ± SEM; **, P < 0.01; ****, P < 0.0001; p-values determined by Wilcoxon signed-rank test (a-d).

As previously, to investigate these changes in global atMBCs, we next examined the frequency of atMBCs according to clinical parameters. There was no difference in the frequency of global atMBCs when stratified by HBsAg load, serum ALT levels or disease phase (Figure 4.7A-C); however, there was a trend towards a higher frequency of atMBCs in patients with high viral loads indicative of hepatitis (≥ $2x10^6$IU/ml; defined by EASL guidelines; Figure 4.7D), suggesting that ongoing viral replication contributes to their accumulation. In line with this finding, the frequency of global atMBCs appeared to be higher in patients with acute HBV infection, and decreased when re-sampled at resolved time points, although this was not significant (Figure 4.7E).

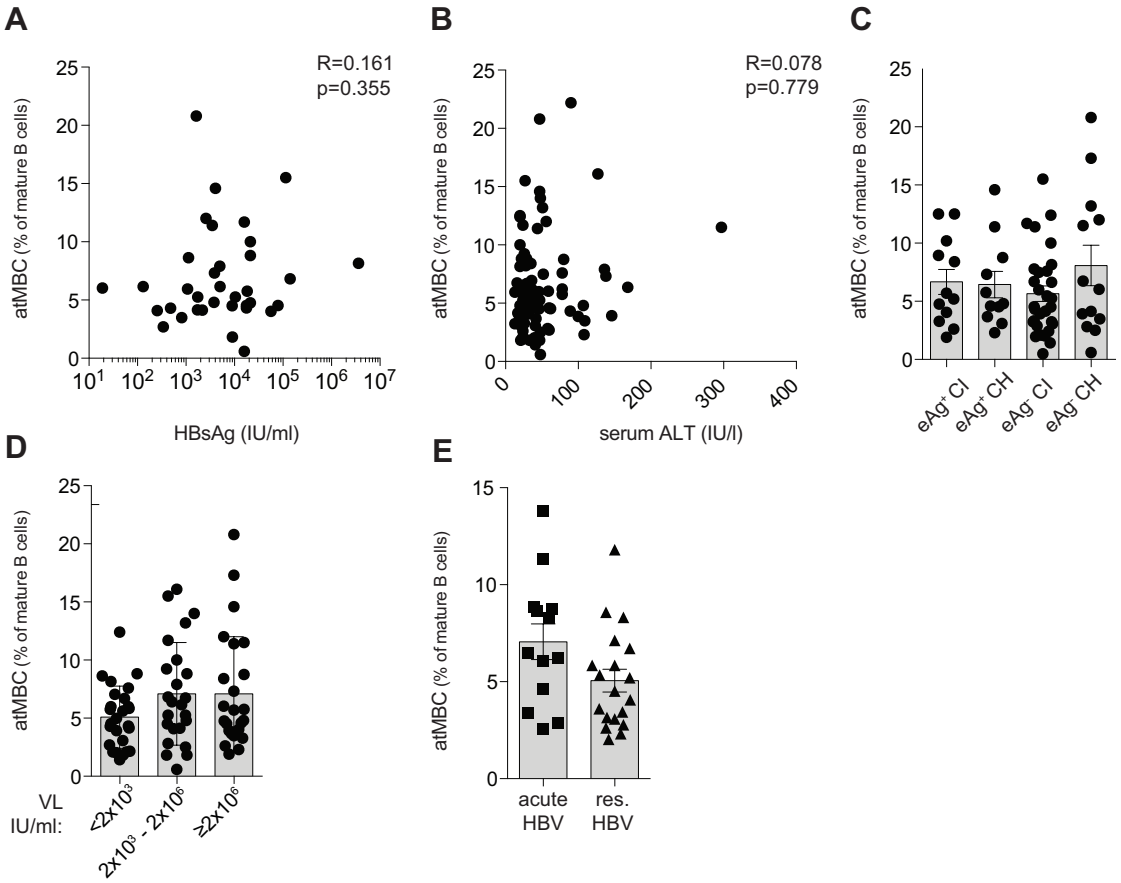

Figure 4-7 Association of global atMBCs according to clinical parameters

A. Frequency of global atMBC correlated with HBsAg (IU/ml; n = 57) or **B.** serum ALT (n=84). **C.** Frequency of global atMBC stratified by disease phase, as defined in Fig. 4.4: n=12 'eAg$^+$ CI'; n=11 'eAg$^+$ CH'; n=30 'eAg$^-$ CI'; and n=13 'eAg$^-$ CH'; and **D.** stratified by viral load: n=26 with HBV DNA <$2x10^3$ IU/ml; n=24 with HBV DNA <$2x10^3$ - $2x10^6$ IU/ml; and n=25 with HBV ≥ $2x10^6$ IU/ml. **E.** Analysis of the frequency of global atMBCs between HBV-acute and HBV-resolved time points. Error bars indicate mean ± SEM; p-values were determined by Spearman's Rank correlation (a and b); Kruskal-Wallis test with a Dunn's post hoc test for pairwise multiple comparisons (c and d); and Mann-Whitney *U* test for unpaired data (e); where no p-value is shown, significance was not reached.

To further investigate these results, we again extended our analysis to longitudinal data sets from three patients with acute-resolving infection. This revealed a tendency of global atMBCs to decrease in line with resolution of disease (Figure 4.8A). atMBC numbers appeared highest prior to the peak of serum ALT in two out of three patients. However, it is difficult to assess the starting levels of atMBCs in acute-resolving disease due to the late presentation of patients in clinic, typically around 10-12 weeks following inoculation, by which point the burden of viral load has already been cleared. Instead, analysis of a chronically infected patient experiencing a flare in disease activity allowed us to examine changes in atMBCs frequencies in accordance with increasing viral load and ALT levels. This suggested that the frequency of atMBCs peaked prior to the rise in ALT levels and viraemia, and declined following the flare (Figure 4.8B). Combined, these data suggested that the expansion of global atMBCs is in part associated with disease activity and high levels of viral replication.

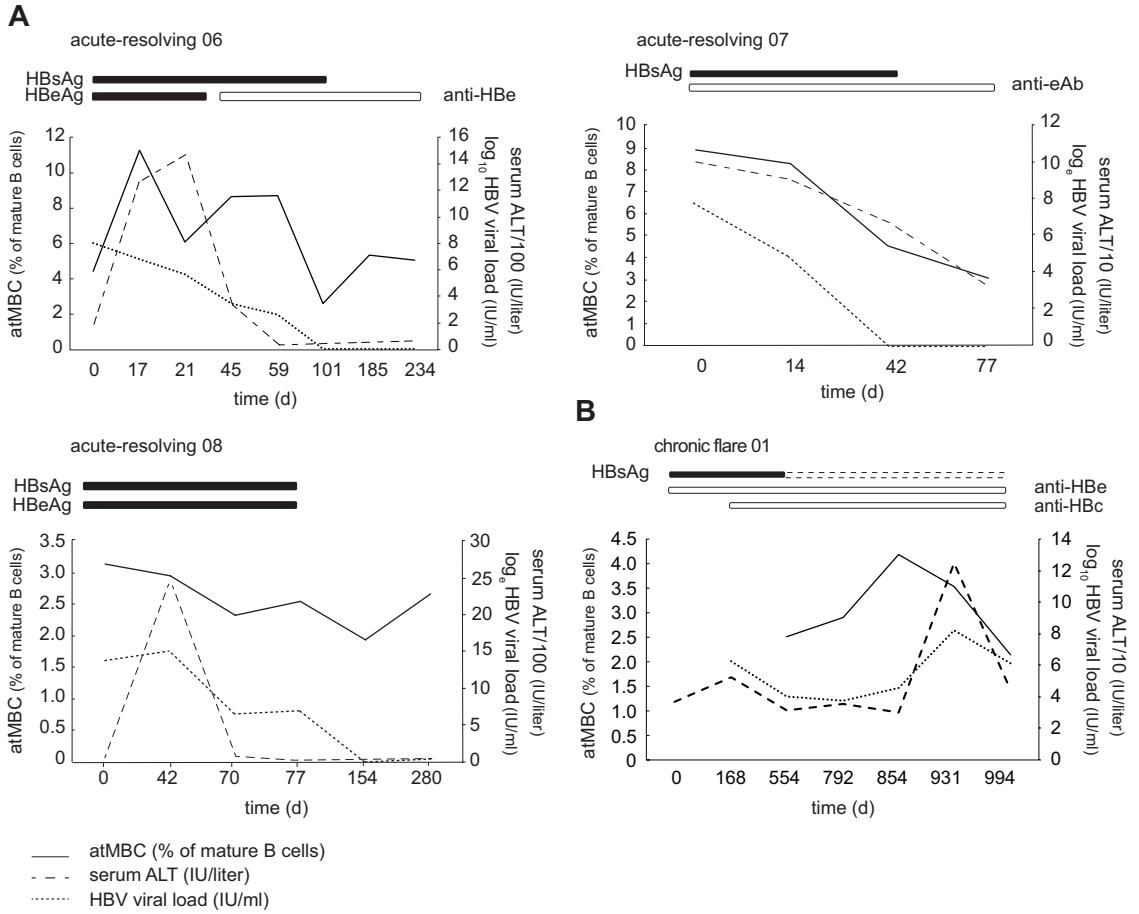

Figure 4-8 Longitudinal sampling of global atMBCs during acute-resolving HBV-infection

Longitudinal analysis of global atMBCs (% of $CD20^+CD19^+CD10^-$) during **A.** acute-resolving infection and **B.** a patient with CHB undergoing hepatic flare. Percentage of atMBCs (black line) are plotted in relation to viral load (dotted line; IU/ml), serum ALT (dashed line; IU/litre) and serological status as indicated by the bars.

4.3.3 atMBCs are T-bet^{hi} and express markers indicative of impaired homing to lymphoid sites

The differentiation of CD27^{-}CD21$^{low/-}$ B cells has been previously associated with induction of T-bet following chronic exposure to antigen (section 4.1.5) (Myles et al., 2017). In line with this, T-bet expression was increased in atMBCs compared to counterpart cMBCs from within the same individual (Figure 4.9A). Accordingly, atMBCs represented the majority subtype within the T-bet^{hi} fraction (mean = 36%; Figure 4.9B). Gates delineating T-bet$^{hi/int/low}$ fractions were set through comparison to the total lymphocyte fraction (representative example in Figure 4.9b): here, T-bet shows clear segregation, in line with data showing T-bet expression gradients in B and T cells (Knox et al., 2014; Obeng-Adjei et al., 2017).

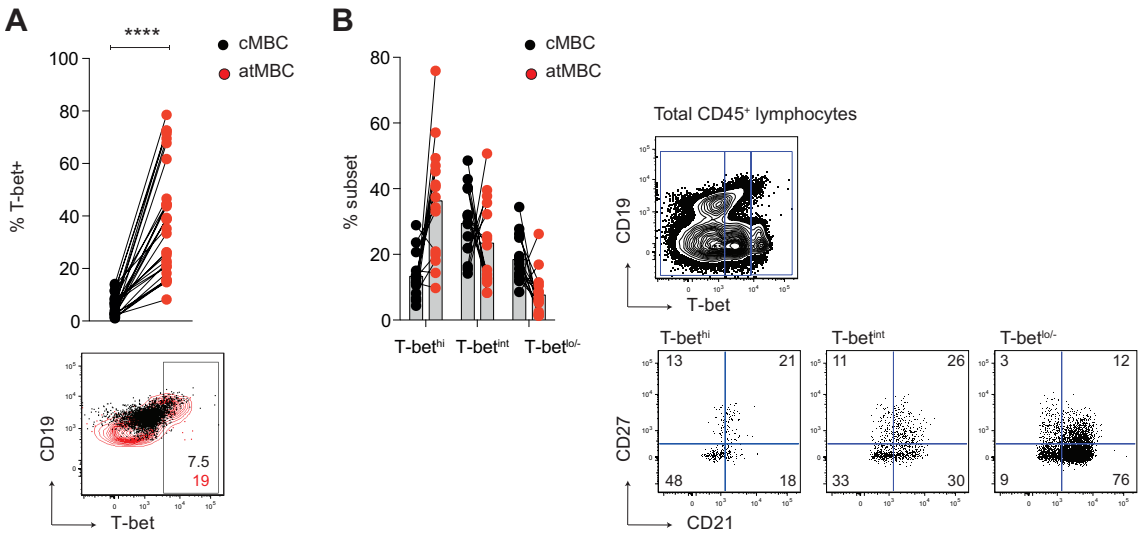

Figure 4-9 atMBCs in patients with CHB have upregulated expression of T-bet

Representative examples and cumulative data. **A.** Expression of T-bet on global atMBC and cMBC (%; n=30). **B.** Percentage of memory B cell subsets within T-bet^{hi}, Tbet^{int} or Tbet^{lo} fractions (pre-gated on CD20^{+}CD19^{+}CD10^{-}; n=15). Gates were drawn on total CD45^{+} lymphocytes, as shown. Error bars indicate means ± SEM; ****, P < 0.0001; p-values determined by Wilcoxon signed-rank test (a).

CD27^{-}CD21$^{low/-}$ B cells that are expanded in autoimmunity and ageing have been demonstrated to highly express the integrin CD11c, associated with their accumulation in inflammatory sites (Karnell et al., 2017). Accordingly, we next analysed the expression of CD11c, alongside other chemokine receptors responsible for directing the migration of B cells. atMBCs in CHB demonstrated an increased expression of CD11c when compared to cMBCs and to a matched isotype/FMO (Figure 4.10A). Expression of CD11c, and the liver homing chemokine receptor CXCR3, was increased within T-bet^{int}/T-bet^{hi} cells (Figure 4.10B), as previously described, indicative of localisation of T-bet^{+} B cells within inflammatory tissue sites (Karnell et al., 2017; Knox et al., 2017; Moir et al., 2008; Obeng-Adjei et al., 2017).

In contrast, expression of the lymph node homing receptor CXCR5 was decreased on atMBCs relative to cMBCs, suggestive of a decreased propensity to home to B cell follicles of lymph nodes (Figure 4.10C).

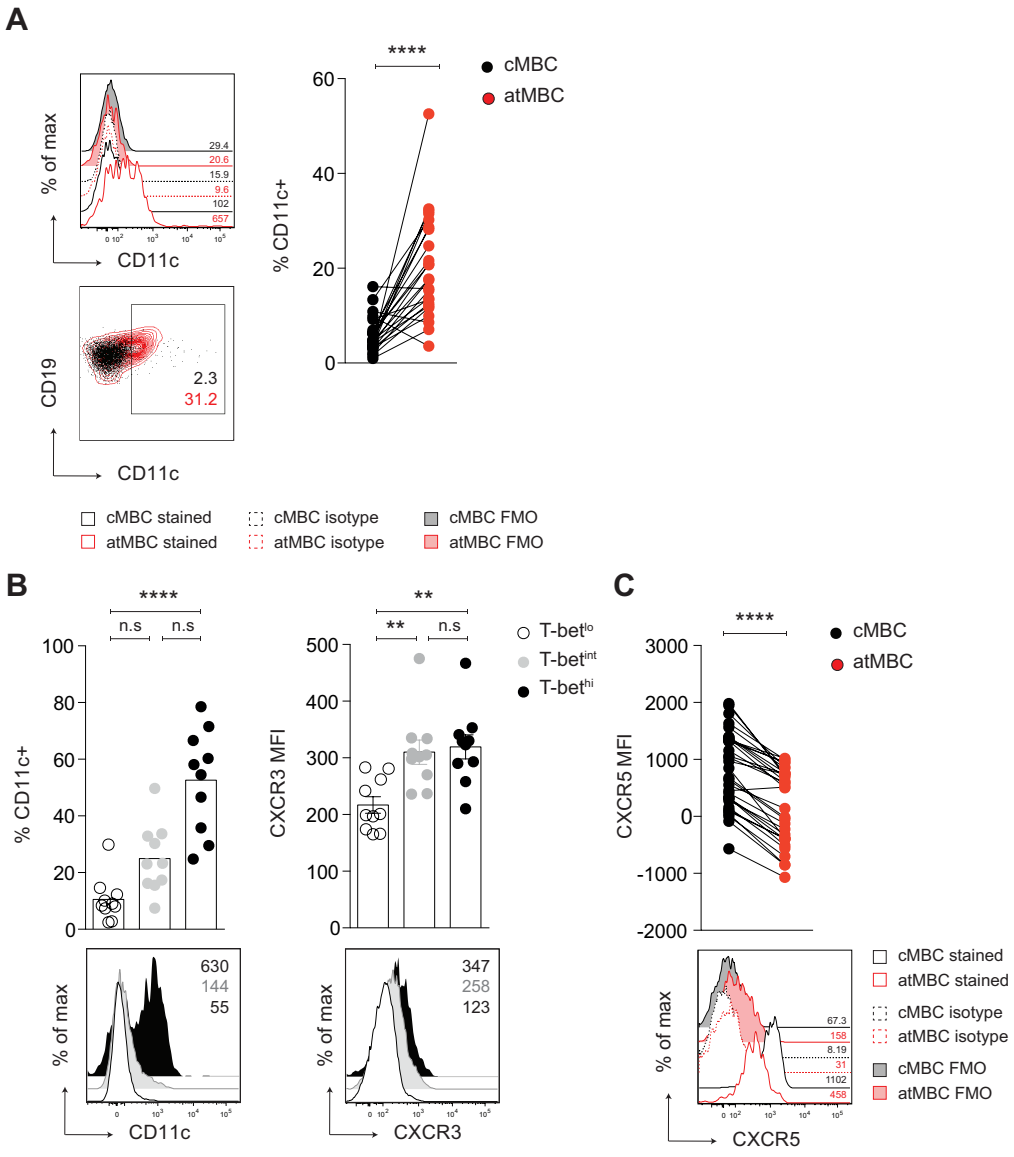

Figure 4-10 Homing receptor profile on memory B cell subsets

Represenative examples and cumulative data. **A.** Expression of CD11c (%; n=24) on global atMBC and cMBC in patients with CHB (solid lines), compared to FMO (shaded) and isotype control (dashed lines). **B.** Expression of CD11c (%; n=10) and CXCR3 (MFI; n=10) on Tbet^{lo} (white), Tbet^{int} (grey) or T-bet^{hi} (black) atMBCs. **C.** Expression of CXCR5 (MFI; n=33) on global atMBCs and cMBCs in patients with CHB. Number displayed in representative plots indicate corresponding MFI. Error bars indicate mean ± SEM; **, P < 0.01; ****, P < 0.0001; p-values determined by Wilcoxon signed-rank test (a, and c); and Kruskal-Wallis test with a Dunn's post hoc test for pairwise multiple comparisons (b).

In addition to decreased expression of lymphoid homing receptors, atMBCs also demonstrated reduced expression of co-stimulatory molecules, CD80 and CD40, compared to counterpart cMBCs (Figure 4.11A), suggestive of diminished activation and T cell help within lymphoid tissue. Accordingly, we next examined the levels of class-switching in memory B cell subsets. This was determined by *ex vivo* staining for IgM and IgD; cells that lack expression of both immunoglobulin isotypes can be deemed a class-switched population. The proportion of atMBCs that had undergone class-switching, and had down-regulated expression of IgM and IgD, was intermediate between cMBC and naïve B cells (Figure 4.11B) and in agreement with previous studies (Portugal et al., 2015). Activated memory B cells ($CD27^{+}CD21^{low/-}$) displayed the highest level of class-switching, in line with their role as activated effector cells (Figure 4.11B).

Combined, these data indicate that the expression profile of atMBCs does not support appropriate homing to secondary lymphoid organs, but may instead favour migration to inflamed tissue sites, such as HBV-infected liver. Impaired interactions with T helper cells, demonstrated by decreased expression of co-stimulatory molecules, may further impact on the functioning of these cells, as indicated by reduced levels of class-switching.

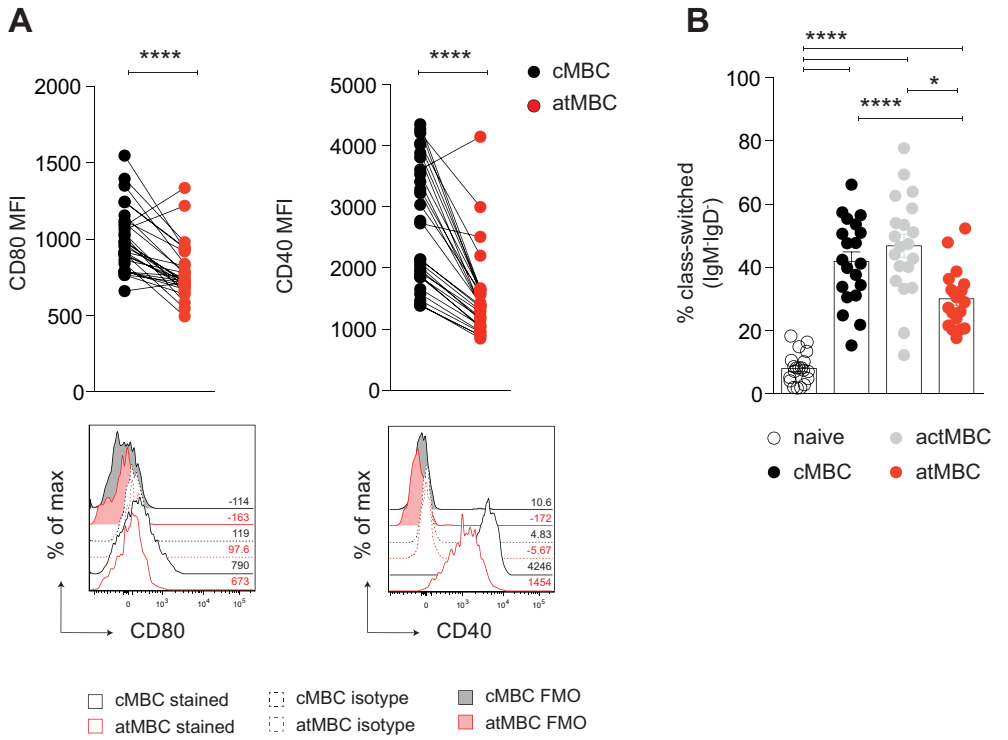

Figure 4-11 Expression of co-stimulatory molecules on atMBCs compared to cMBCs

A. Representative examples and cumulative data: expression of CD80 (MFI; n=30) and CD40 (MFI; n=30) on global atMBCs and cMBCs in patients with CHB, compared to FMO (shaded) and isotype control (dashed line). **B.** Frequency of class-switched cells ($IgM^{-}IgD^{-}$) as a percentage of naïve, cMBCs, actMBCs and atMBCs (n=39 patients with CHB). Error bars indicate mean ± SEM; *, $P < 0.05$; ****, $P < 0.0001$; p-values determined by Wilcoxon signed-rank test (a); and Kruskal-Wallis test with a Dunn's post hoc test for pairwise multiple comparisons (b).

4.3.4 Global atMBCs in CHB express an array of inhibitory receptors

Upon activation, lymphocytes transiently express co-inhibitory molecules that help to regulate responses - activated cells must overcome these signals in order to exert their full effector function. In chronic viral infections, virus-specific lymphocytes are often restrained by the persistent expression of co-inhibitory molecules, that are induced as a result of chronic antigenic stimulation (outlined in Chapter 1.3.3.1). Previous data have shown that atMBCs can be constrained by high levels of inhibitory receptors expression (Moir et al., 2008; Portugal et al., 2015). Therefore, we were interested to examine the expression of co-inhibitory signals on atMBCs as a possible mechanism by which antigen-specific B cells are suppressed in patients with CHB.

To investigate this, we examined the relative expression levels of receptors shown to negatively regulate B cell activation. Expression of both B and T lymphocyte attenuator (BTLA) and CD22 – receptors involved in the negative regulation of BCR signalling - were significantly increased on atMBCs compared to counterpart cMBCs from within the same individual and above staining by a matched isotype (Figure 4.12A). Two additional receptors, FcγRIIB and FcR-like receptor-5 (FcRL5; CD307), which bind to the Fc portion of antibodies (Franco et al., 2013; Karnell et al., 2014), were also significantly enriched on atMBCs (Figure 4.12B). Finally the prototypic T cell checkpoint inhibitor, PD-1 (Zehn et al., 2016), was highly expressed on a subset of atMBCs but showed minimal expression on cMBCs (Figure 4.12C). PD-1 expression was particularly enriched on T-bet^{hi} atMBCs, at odds with the description of T-bet^{hi} B cells as key antiviral effector cells (Figure 4.12D).

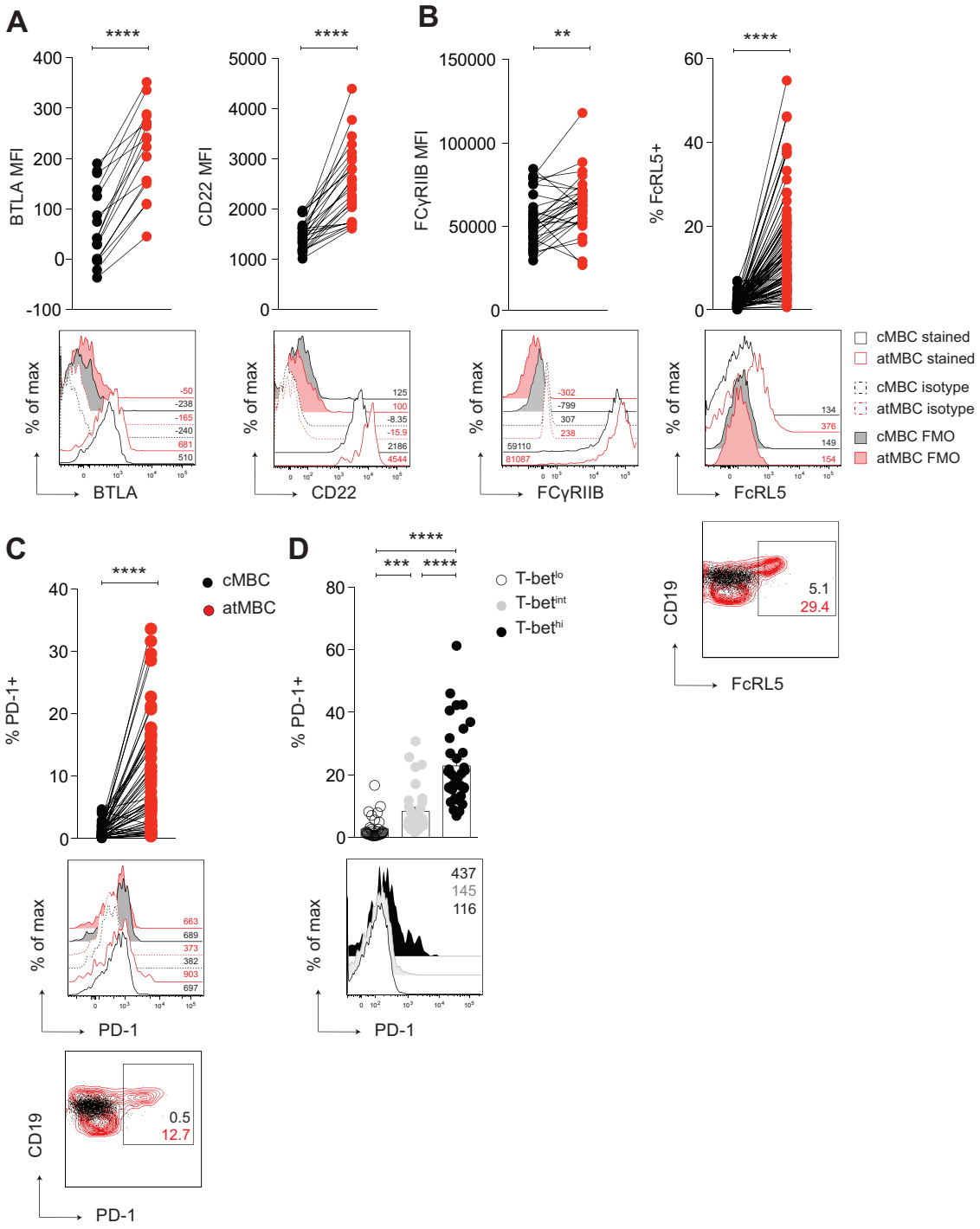

Figure 4-12 Expression of inhibitory receptors on atMBCs compared to cMBCs

Representative examples and cumulative data. Expression of **A.** BTLA (MFI; n=16) and CD22 (MFI; n=26); **B.** FcγRII1B (CD32b; MFI; n=30) and FcRL5 (%; n=83); and **C.** PD-1 (%; n=55) on atMBCs and cMBCs in patients with CHB, compared to FMO (shaded) and isotype control (dashed line). **D.** Expression of PD-1 (%; n=20) on Tbet^{lo} (white), Tbet^{int} (grey) or T-bet^{hi} (black) atMBCs. Error bars indicate mean ± SEM; **, P < 0.01; ***, P < 0.001; ****, P < 0.0001; p-values determined by Wilcoxon signed-rank test (a, b and c); and Kruskal-Wallis test with a Dunn's post hoc test for pairwise multiple comparisons (d).

4.3.5 PD-1 expression is associated with antigen experience

As receptor expression was not ubiquitous to all atMBCs, we next investigated whether there was a subset within $CD27^{-}CD21^{low/-}$ B cells that preferentially expressed high levels of inhibitory receptors. To do so, we employed dimension reduction analysis on concatenated flow cytometric data of global B cells, using t-distributed stochastic neighbour embedding (tSNE). This analysis facilitates visualisation of high-dimensional data by grouping cells according to the similarity of cell surface marker expression. In doing so, tSNE is able to recognise associations between markers that may otherwise be missed using conventional gating (Thomas and Pallett, 2018).

Visualisation by tSNE revealed that atMBCs clustered as two discrete populations when defined by the expression of FcRL5 in addition to the $CD27^{-}CD21^{low/-}$ phenotype (Figure 4.13A). This segregation of $FcRL5^{+}$atMBCs was driven by differential expression of IgM. As a result, atMBCs could be sub-divided into $IgM^{+/-}IgD^{+}$ cells and $IgM^{-}IgD^{-}$ cells, likely representing a naïve-like, non-class-switched sub-type and class-switched B cells respectively. Interestingly, when stratifying according to PD-1, we found that expression across all B cells was concentrated in $CD27^{-}CD21^{low/-}$ B cells, particularly on those that were class-switched ($IgM^{-}IgD^{-}FcRL5^{+}$ B cells lacking CD21 and CD27), implicating PD-1 as a hallmark of antigen-experienced atMBC in CHB (Figure 4.13A).

Through examining healthy controls, HBV-inactive carriers (defined as having a viral load <2000IU/ml) and patients with elevated viral loads (defined as ≥ 2000IU/ml), we showed that the subset of $PD\text{-}1^{+}$ atMBCs was increased in patients with active HBV-infection; although some atMBC in healthy controls and low-level HBV carriers expressed PD-1, this proportion was more than doubled in HBV carriers with higher viral loads >2000IU/ml (Figure 4.13B).

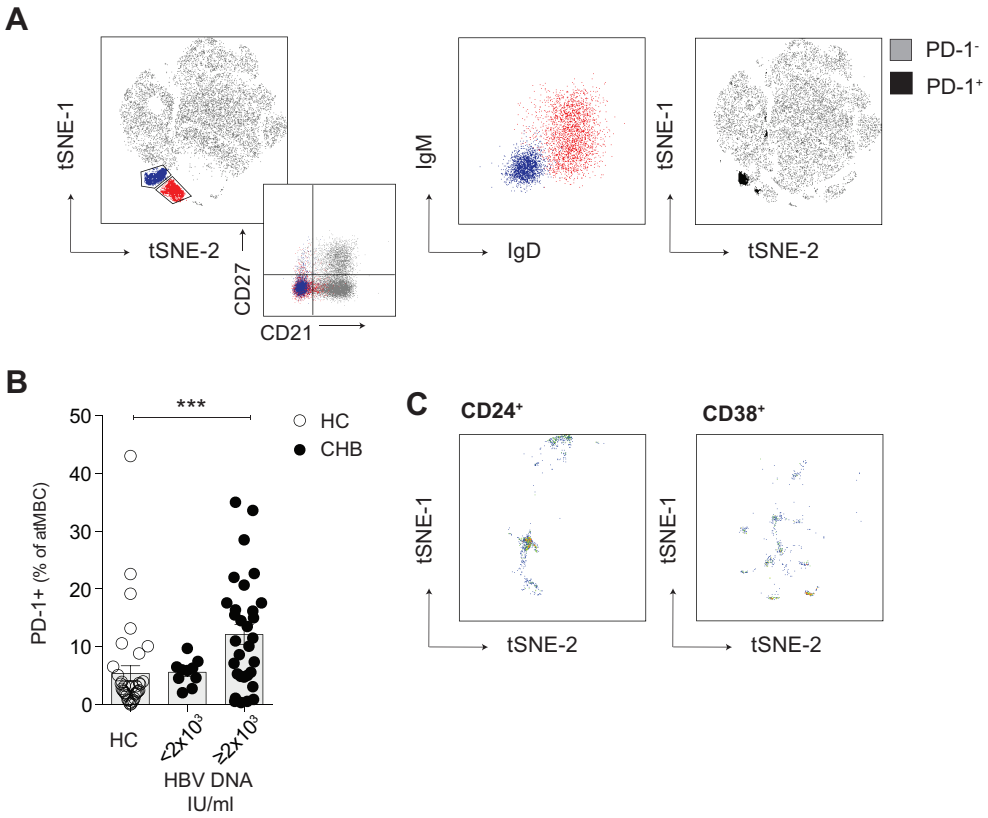

Figure 4-13 Visualisation of PD-1 expression on B cell subsets

tSNE analysis courtesy of Dr. Leo Swadling. **A**. Dimension reduction analysis visualised using tSNE identifying discrete populations of $IgM^{+}IgD^{+/-}$ (red) and $IgM^{-}IgD^{-}$ (blue) atMBCs based on the expression profile $CD21^{-}CD27^{-}$ $FcRL5^{+}$. PD-1 expression on B cells was concentrated within $IgM^{-}IgD^{-}$ atMBCs (purple cluster). tSNE analysis was performed on the expression data for the markers IgD, CD21, CD80, BAFF-R, CD10, CD11c, CD27, FcRL5, CD20, IgM, PD-1, CD38, CD24 as measured by flow cytometry on $CD19^{+}$ events concatenated from patients with CHB (n=8) and vac HC (n=8). **B.** Frequencies of $PD\text{-}1^{+}$ atMBC stratified by viral load (IU/ml): n=10 with HBV DNA $<2x10^{3}$; n=31 with HBV DNA $\geq 2x10^{3}$) and compared to healthy controls (HC; n=37). **C.** Clusters of $CD24^{+}FcRL5^{-}CD27^{-}CD21^{low/-}$ B cells and $CD38^{+}FcRL5^{-}CD27^{-}CD21^{low/-}$ B cells. Error bars indicate mean ± SEM; ***, $P < 0.001$; p-values determined by Kruskal-Wallis test with a Dunn's post hoc test for pairwise multiple comparisons (b).

tSNE analysis also identified additional populations of $CD38^{-}CD24^{+}$ and $CD38^{+}CD24^{-}$ atMBCs, although these were less discrete and did not express FcRL5, nor PD-1 (Figure 4.13C). $CD38^{+}$ atMBCs in particular may represent a subset of antigen-experienced effector cells postulated to exist within $CD27^{-}CD21^{low/-}$ fractions (Swain et al., 2017).

4.3.6 HBsAg-specific B cells in patients are enriched for markers of atMBCs

To investigate whether these finding translate from global atMBCs to HBsAg-specific B cells in patients with CHB, we next examined the inhibitory receptor profile on HBsAg-bait binding cells identified directly *ex vivo*. Both FcRL5 and PD-1 were consistently enriched on HBsAg-specific B cells compared to their global B cell counterparts (Figure 4.14A, B). In addition, FcRL5- and PD-1-expressing HBsAg-specific B cells were increased in patients with CHB relative to vaccinated controls (Figure 4.14A, B). Similarly, B cells expressing high levels of the transcription factor T-bet were increased by more than 2-fold in the HBsAg-specific compartment compared to global B cell compartment in patients with CHB, and compared to HBsAg-specific B cells in vaccinated healthy controls (Figure 4.14C). Accordingly, HBsAg-specific B cells also demonstrated elevated levels of CD11c expression and less CXCR5 compared to controls, suggestive of a preference for inflamed, non-lymphoid tissue (Figure 4.14D, E). Finally, CD40 expression was decreased on HBsAg-specific B cells from patients with CHB compared to vaccinated healthy controls, but showed no increased expression on HBsAg-specific compared to global B cells (Figure 4.14F). Combined, these data suggest that HBV-infection imparts significant changes on both global and HBsAg-specific B cells that may impact on their downstream function.

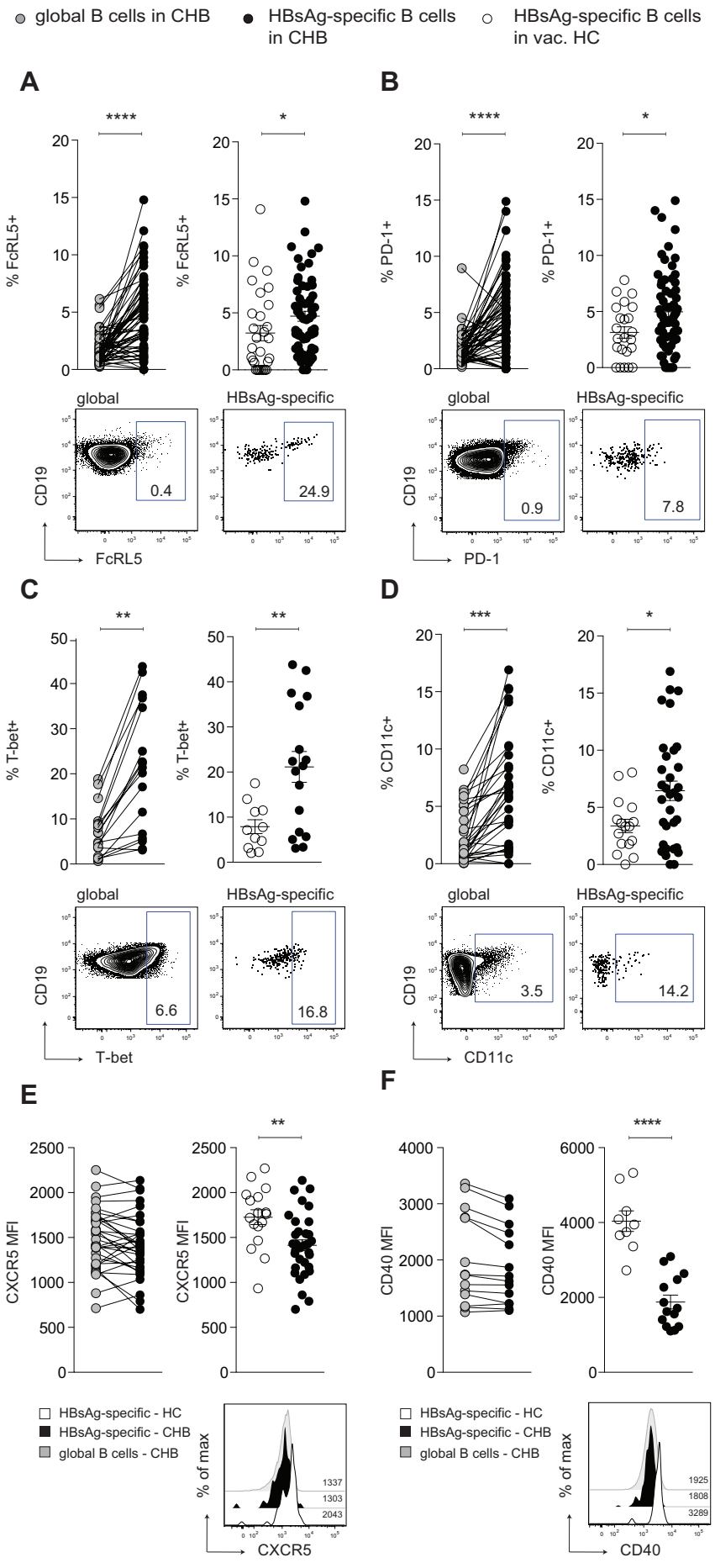
global B cells in CHB
HBsAg-specific B cells in CHB
HBsAg-specific B cells in vac. HC
A
B
C
D
E
F
% FcRL5+
% PD-1+
% T-bet+
% CD11c+
CXCR5 MFI
CD40 MFI
% of max
global
HBsAg-specific
CD19
FcRL5
PD-1
T-bet
CD11c
CXCR5
CD40
0.4
24.9
0.9
7.8
6.6
16.8
3.5
14.2
HBsAg-specific - HC
HBsAg-specific - CHB
global B cells - CHB
1337
1303
2043
1925
1808
3289

Figure 4-14 Phenotypic analysis of HBsAg-specific B cells in patients with CHB compared to HBV-vaccinated healthy controls

Representative examples and cumulative data: paired analysis of marker expression on HBsAg-specific B cells (black) compared to global B cells (grey) from within the same patient with CHB, and unpaired comparison of HBsAg-specific B cells in patients with CHB and vaccinated healthy controls (white). Expression levels of **A.** FcRL5 (%; n=60 patients with CHB; n=29 vac HC); **B.** PD-1 (%; n=66 patients with CHB; n=23 vac HC); **C.** T-bet (%; n=17 patients with CHB; n=11 vac HC); **D.** CD11c (%; n=33 patients with CHB; n=16 vac HC); **E.** CXCR5 (MFI; n=33 patients with CHB; n=17 vac HC); and **F.** CD40 (MFI; n=15 patients with CHB; n=9 vac HC). Error bars indicate mean ± SEM; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$; p-values were determined by Wilcoxon signed-rank test for paired data and Mann-Whitney *U* test for unpaired data.

4.3.7 atMBCs exhibit markers of attenuated BCR signalling

Due to the dysfunctional phenotype of atMBCs and changes within HBsAg-specific B cells demonstrated *ex vivo*, we next examined the functional status of atMBCs compared to conventional cMBCs, aiming to understand how an enrichment of B cells with an atMBC phenotype, in conjunction with a decrease in cMBCs, within HBsAg-specific B cells may, contribute to impaired antiviral responses in CHB. Previous work has indicated that genes encoding BCR signalling and associated co-stimulatory molecules are down-regulated in atMBCs (Sullivan et al., 2015). This has been, in part, attributed to elevated expression of inhibitory receptors: FcRL5 expressing B cells are reported to be deficient in their ability to secrete cytokine and antibody in response to BCR stimulation, with FcRL5 shown to functionally impair memory B cells through direct suppression of B cell receptor signalling (Davis, 2015; Haga et al., 2007; Portugal et al., 2015; Sullivan et al., 2015; Zhu et al., 2013).

BCR signalling is central to regulating proliferation and effector function in B cells. Signal propagation from the BCR is mediated by multiple pathways, chiefly via phospholipase C-γ2 (PLC-γ2) or phosphatidalyinositol-3-kinase (PI3K) pathways. PLC-γ2 activation, in particular, results in the accumulation of cytosolic calcium, which in turn induces the expression of transcription factors that contribute to the activation of B cells and determination of cell fate (Hogan et al., 2003).

Therefore, to test the responsiveness of these cells to antigen, we measured calcium mobilisation following BCR engagement as a gauge of B cell signalling required for subsequent differentiation and effector function in B cells (Portugal et al., 2015). B cells were magnetically isolated from the PBMCs of patients with CHB and loaded with Fluo-4 AM calcium dye, prior to stimulation with $F(ab')_2$ anti-IgM/IgG/IgA specific antibodies or ionomycin. Calcium mobilisation was measured by flow cytometry. 5 x 10^5 B cells were stimulated and run at a high threshold to limit fluctuations in cell number that may otherwise account for variations in fluorescence and ensure that a continuous stream of each subset was recorded (Figure 4.15A, B). Ca^{2+} mobilisation was recorded over time and analysed according to B cell subset (Figure 4.15B). BCR crosslinking induced robust Ca^{2+}

mobilisation in cMBCs and activated memory B cells (actMBCs), in spite of the low frequency of actMBCs (Figure 4.15C). In contrast, Ca^{2+} mobilisation was markedly reduced in atMBCs following BCR-ligation, as shown by the difference in peak median fluorescence between the stimulated and unstimulated time points (Figure 4.15C). Naïve B cells showed weaker and delayed Ca^{2+} mobilisation compared to actMBCs and cMBCs, but above that of atMBCs, in line with data suggesting that naïve B cells require a greater threshold of activation following BCR engagement (Moens et al., 2016). When stimulated with ionomycin, overriding the BCR, all subsets demonstrated comparable ability to mobilise Ca^{2+}, suggesting that impairment may be overridden by certain signals (Figure 4.15C).

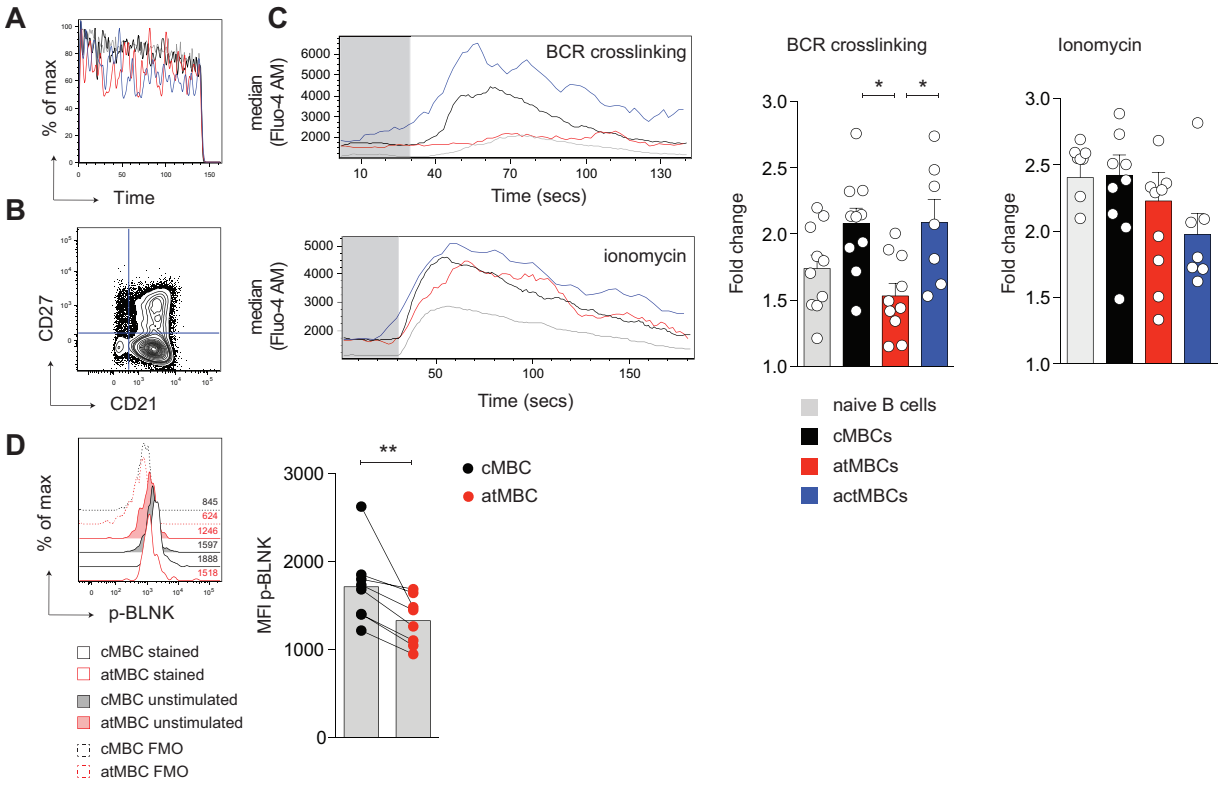

Figure 4-15 Ca^{2+} mobilisation in memory B cell subsets following BCR cross-linking

A. Representative plot demonstrating the frequency of B cell subsets over the time course of BCR stimulation. **B**. Representative plot of staining identifying B cell subsets following BCR stimulation. **C.** Representative flow cytometric analysis of Ca^{2+} mobilisation (Fluo-4 AM; median fluorescence intensity) over time (s) in purified B cells after stimulation with either $F(ab')_2$ anti-IgG/IgA/IgM (anti-BCR; 50ug/ml) or ionomycin (1ug/ml) (n=10 patients with CHB). Basal fluorescence prior to stimulation is shaded in grey. Summary plot shows the difference in mean fluorescence intensity of Fluo-4 AM upon stimulation in B cell subsets compared to baseline. **D.** Expression of phosphorylated-BLNK (MFI; p-BLNK) in global B cells after crosslinking with $F(ab')_2$ anti-IgG/IgA/IgM for 30 s (anti-BCR; n=8). Error bars indicate mean ± SEM; *, $P < 0.05$; **; p-values determined by Kruskal-Wallis test with a Dunn's post hoc test for pairwise multiple comparisons (c) and Wilcoxon signed-rank test (d).

We next endeavoured to look at pathways upstream of Ca^{2+} mobilisation by examining the phosphorylation status of B-cell linker protein (BLNK), an adaptor molecule important in co-ordinating B cell receptor signalling. Following BCR ligation, BLNK is phosphorylated by Syk and transduces signals that activate PLC-γ2 and Ca^{2+} mobilisation (Ishiai et al., 1999; Kurosaki and Tsukada, 2000; Tan et al., 2001). Levels of phosphorylated-BLNK (p-BLNK) were decreased in atMBCs compared to cMBC upon BCR triggering (Figure 4.15D). Together, these data provide evidence to suggest that atMBCs may have attenuated B cell receptor signalling in response to antigen stimulation. This defect may impair activation of atMBCs, thus impacting on downstream antiviral function. However, more in-depth analysis of downstream messenger proteins is required to elucidate how BCR signalling may be impaired in atMBCs.

4.3.8 atMBCs demonstrate impaired antiviral function

Due to the impaired signalling and inhibitory profile of atMBCs, we next investigated their antiviral function by examining their capacity to secrete key antiviral cytokines in response to both T cell dependent and T cell independent stimulation. It is increasingly recognised that B cells can be an important source of cytokines in a number of settings, including viral infection (Das et al., 2012; Lin et al., 2002; Rosser and Mauri, 2015). In antiviral immune responses, B cell-derived cytokines promote the organisation of lymphoid structures and differentiation of effector and memory T cells (Section 1.4.7.1). However, they can also have direct acting effects on HBV itself (Shen and Fillatreau, 2015); both TNFα and Interleukin-6 (IL-6) have been shown to have potent non-cytolytic antiviral activity in HBV infection (Bouezzedine et al., 2015; Guidotti et al., 1996; Hösel et al., 2009; Palumbo et al., 2015; Xia et al., 2016). The production of these key antiviral cytokines by B cells has, to our knowledge, not yet been determined.

To test this, PBMCs were stimulated using T-cell dependent or independent stimuli in the presence of Brefeldin-A to block egress of cytokines from the endoplasmic reticulum. Cells were then stained for the presence of intracellular cytokines, in combination with B cell phenotypic markers to permit subset identification. Stimulation was limited to 16 hours to avoid sizeable changes to B cell phenotype throughout the duration of the culture period. Crosslinking of the B cell receptor in association with CD40 stimulation demonstrated that $CD27^{-}CD21^{low/-}$ cells secrete significantly less TNFα and IL-6 compared to $CD27^{+}CD21^{+}$ cMBCs (Figure 4.16A, B), indicative of impaired antiviral function.

Previous reports have described atMBCs that do not respond to BCR signalling, but remain receptive to triggering through Toll-like receptors (TLR) (Portugal et al., 2017). Thus, we next assessed the ability of these cells to respond to TLR signals, independently of BCR signalling. TLR-7 is highly expressed on memory B cell subsets (Bernasconi et al., 2003), where it can bind viral single stranded RNA (Sepehri et al., 2016) and induce the secretion of cytokines, including

IL-6, IL-8 and IL-10 (Barr et al., 2007; Glaum et al., 2009; Hanten et al., 2008). atMBCs stimulated with R848 (TLR-7/8 agonist) were again significantly impaired compared to cMBCs in their ability to produce both cytokines (Figure 4.16A, B). Both atMBCs and cMBCs showed reduced cytokine production in response to BCR triggering relative to TLR stimulation; however, this was more marked on atMBCs, in line with their attenuated BCR signalling. Overal, these data suggest that atMBCs are impaired in their ability to secrete important antiviral cytokines, in line with previous reports (Portugal et al., 2015), and are hypo-responsive to typical B cell stimuli. Efficient activation of atMBCs may therefore require a combination of multiple stimuli.

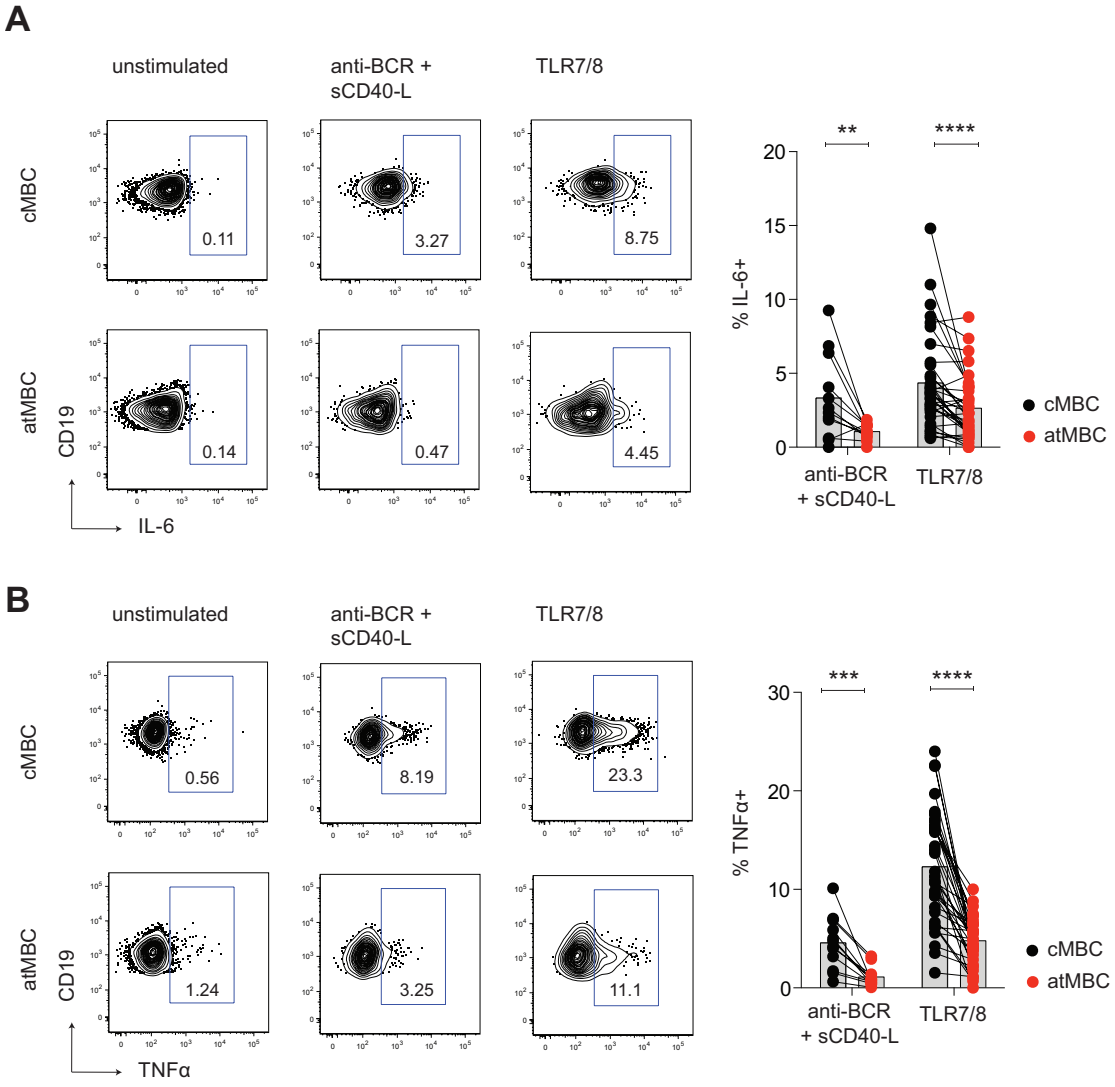

Figure 4-16 Antiviral cytokine production by memory B cell subsets

Intracellular cytokine staining for **A.** IL-6 and **B.** TNFα in atMBC and cMBC after stimulation with F(ab')$_2$ anti-IgG/IgA/IgM and CD40-ligand (anti-BCR; sCD40-L; n=10 patients with CHB) and R848 (Resiquimod; TLR7/8 agonist; 1ug/ml; n=35 patients with CHB) for 16-18hrs. Frequencies are presented minus a paired unstimulated control. Frequency is presented minus a paired unstimulated control. Error bars indicate mean ± SEM; **, P < 0.01; ***, P < 0.001; ****, P < 0.0001; p-values were determined by Wilcoxon signed-rank test (a and b).

4.3.9 atMBCs show impaired differentiation into antibody secreting cells

The differentiation of memory B cells into antibody-secreting plasma cells is a key arm of B cell immunity in viral infections. Data discussed in the previous chapter showed that B cells from patients with CHB had impaired capacity to differentiate to anti-HBs secreting cells, detectable by ELISPOT (compared to healthy HBV-vaccinated controls), and did not produce protective levels of anti-HBs IgG *in vitro*. To understand whether this can be attributed to an expansion of atMBCs in patients with CHB, we next isolated matched numbers of atMBCs and cMBCs and compared their capacity to differentiate into ASCs. FACs-sorted cells were first activated using a TLR-9 agonist (CpG-B), before being cultured with cytokines that promote plasma cell formation (IL-2, IL-15, IL-21, IL-6 and IFNα), as in Figure 3.4. Differentiation was determined post-culture by flow cytometry, with ASCs defined as $IgD^{-}CD38^{hi}CD20^{low}CD27^{+}CD138^{hi/int}$ (Figure 4.17A). Due to limitations related to FACS-sorting of category two hazard material, the majority of this analysis was performed on healthy control samples. For comparison, a matched number of cMBCs were activated and cultured in complete RPMI only (Figure 4.17A bottom panel), which showed markedly impaired differentiation, as expected.

Despite starting with the same number of cells, fewer atMBCs survived and acquired an ASC phenotype, compared to cMBCs from the same donor (Figure 4.17A, B). Staining for live cells using a fixable live/dead stain revealed that the viability of cultured atMBCs was significantly decreased relative to cMBCs (representative example in Figure 4.17C). Therefore, not only did atMBCs have a decreased ability to differentiate into ASCs, but they also demonstrated an increased propensity towards apoptosis, as was initially indicated by decreased survival of HBsAg-specific B cells isolated from patients with CHB, shown in Figure 3.9. Interestingly, those atMBCs that were able to survive and differentiate produced robust levels of total IgG, as measured by cytometric bead array, suggesting that a proportion of atMCBs within healthy individuals retain the ability to form functional ASCs (Figure 4.17D). Of note, atMBCs isolated from a patient with CHB (shown in black) showed markedly impaired differentiation to ASCs compared to counterpart cMBCs, in accordance with substantially reduced production of IgG (Figure 4.17B, D).

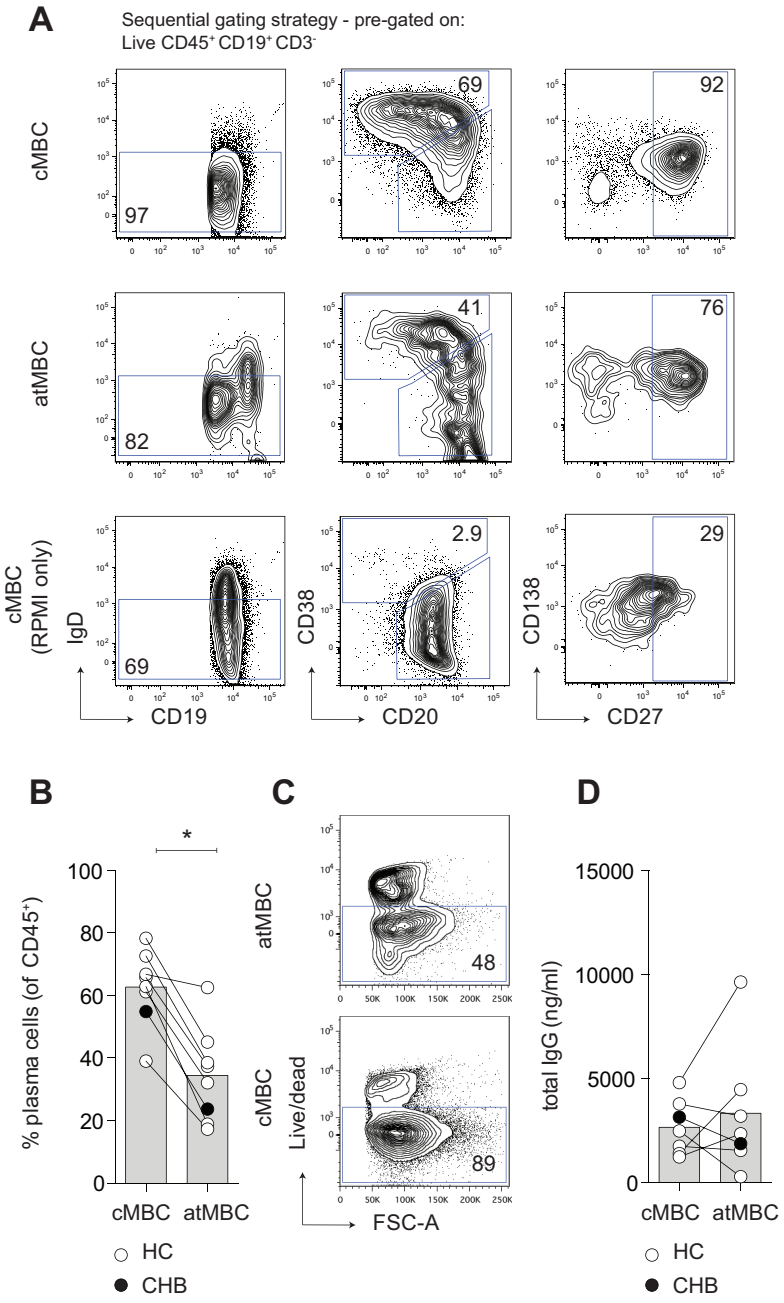

Figure 4-17 ASC differentiation of FACS-sorted memory B cell subsets

A-B. Representative staining and cumulative data: atMBC were FACS sorted from healthy control blood (n=7; white) and one patient with CHB (black) and differentiated into plasma cells alongside a matched number of cMBCs. For comparison, a matched number of cMBCs were cultured in cRPMI without cytokines (bottom panel). **B.** Cumulative data shows the proportion of cells that acquire a plasma cell phenotype. Cells were analysed by flow cytometry, with plasma cells defined as $CD45^+CD19^+CD3^-IgD^-CD38^{hi}CD20^-CD27^+CD138^+$. **C.** Representative example demonstrating impaired survival of atMBCs relative to cMBCs during culture, as assessed using a fixable live/dead dye. **D.** Production of total IgG (ng/ml) measured in culture supernatant by cytometric bead array. Error bars indicate mean ± SEM; *, $P < 0.05$; p-values were determined by Wilcoxon signed-rank test (b and d).

To further investigate apoptosis within atMCBs, we first stained PBMCs directly *ex vivo* for Annexin-V. Annexin-V binds phosphatidylserine which, when expressed on the cell surface, is used as a marker of cells in early stages of apoptosis (Mourdjeva et al., 2005). Positive staining was determined through comparison to an FMO and CD8 T cells (where positive staining was more defined) (Figure 4.18A).

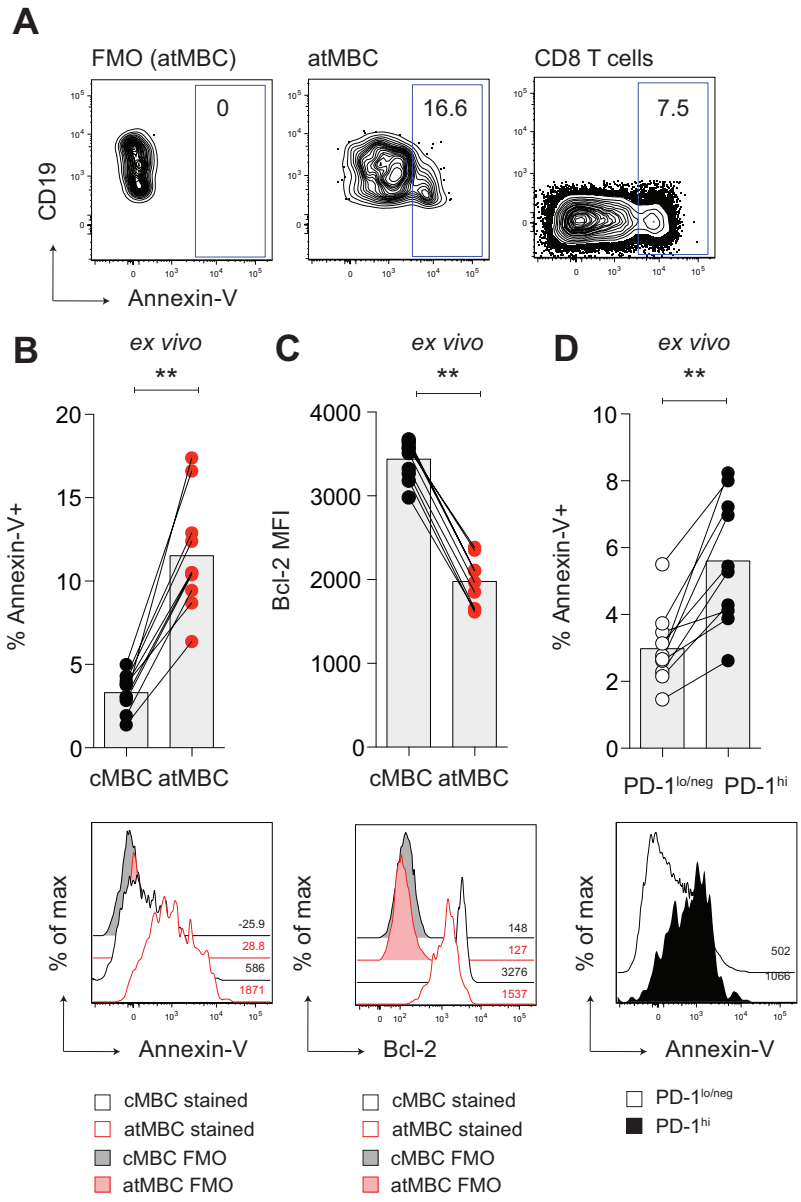

Figure 4-18 *Ex vivo* expression of apoptotic markers on B cell subsets

A. Representative *ex vivo* staining for Annexin-V on B cells on atMBCs, compared to FMO and CD8 T cells.

B. Percentage of cells expressing Annexin-V, stratified by cMBC and atMBC phenotype (n=10 patients with CHB).

C. Expression of Bcl-2 (MFI) on cMBC and atMBCs (n=10 patients with CHB). **D.** Expression of Annexin-V stratified by PD-1 expression on total B cells (n=10 patients with CHB). Error bars indicate mean ± SEM; **, $P < 0.01$; p-values were determined by Wilcoxon signed-rank test (b-d).

Ex vivo atMBCs expressed higher levels of Annexin-V compared to counterpart cMBCs, suggestive of an increased propensity towards apoptosis (Figure 4.18B). In line with this, atMBCs had decreased expression of B cell lymphoma-2 (Bcl-2), an anti-apoptotic protein that protects cells from premature apoptosis (Chipuk et al., 2010; Merino et al., 1994) (Figure 4.18C). Interestingly, Annexin-V expression was also shown to be higher on PD-1^{+} B cells (Figure 4.18D), agreeing with previous reports associating PD-1 with cell death in B cells (Nicholas et al., 2013) and T lymphocytes (Freeman et al., 2006).

The proportion of cells expressing Annexin-V *ex vivo* was low due to the relative viability of immediately thawed PBMCs. To test the predisposition of memory B cells towards apoptosis in culture, we next analysed Annexin-V expression in PBMCs following BCR simulation and CD40-ligation for 96 h. Cells were analysed according to memory B cell subset with Annexin-V gates set according to FMO on counterpart stimulated cells (Figure 4.19A). Following culture for this extended period of time, the proportion of cells with a CD27^{-}CD21$^{low/-}$ phenotype had increased; however, in line with *ex vivo* data, CD27^{-}CD21$^{low/-}$ B cells demonstrated an increased proportion of apoptotic cells compared to their counterpart cMBCs, indicative of increased susceptibility to cell death (Figure 4.19B). Again, Annexin-V expression was increased on PD-1^{+} B cells (Figure 4.19B), suggesting that PD-1 expression may associated with a propensity towards apoptosis. To further investigate this effect of PD-1 expression on apoptosis, we tested the effect of *in vitro* blockade of PD-1 following BCR stimulation and CD40-ligation using a monoclonal antibody. PD-1 blockade significantly decreased the proportion of atMBCs staining positive for Annexin-V, whilst a matched isotype control demonstrated no difference in Annexin-V expression on atMBCs following stimulation (Figure 4.19C). Combined, these data suggest that PD-1^{hi} atMBCs exhibit increased propensity towards apoptosis, along with impaired differentiation to critical effector cells. This impaired survival and differentiation capacity of atMBCs may underscore the diminished production of anti-HBs in patients with CHB.

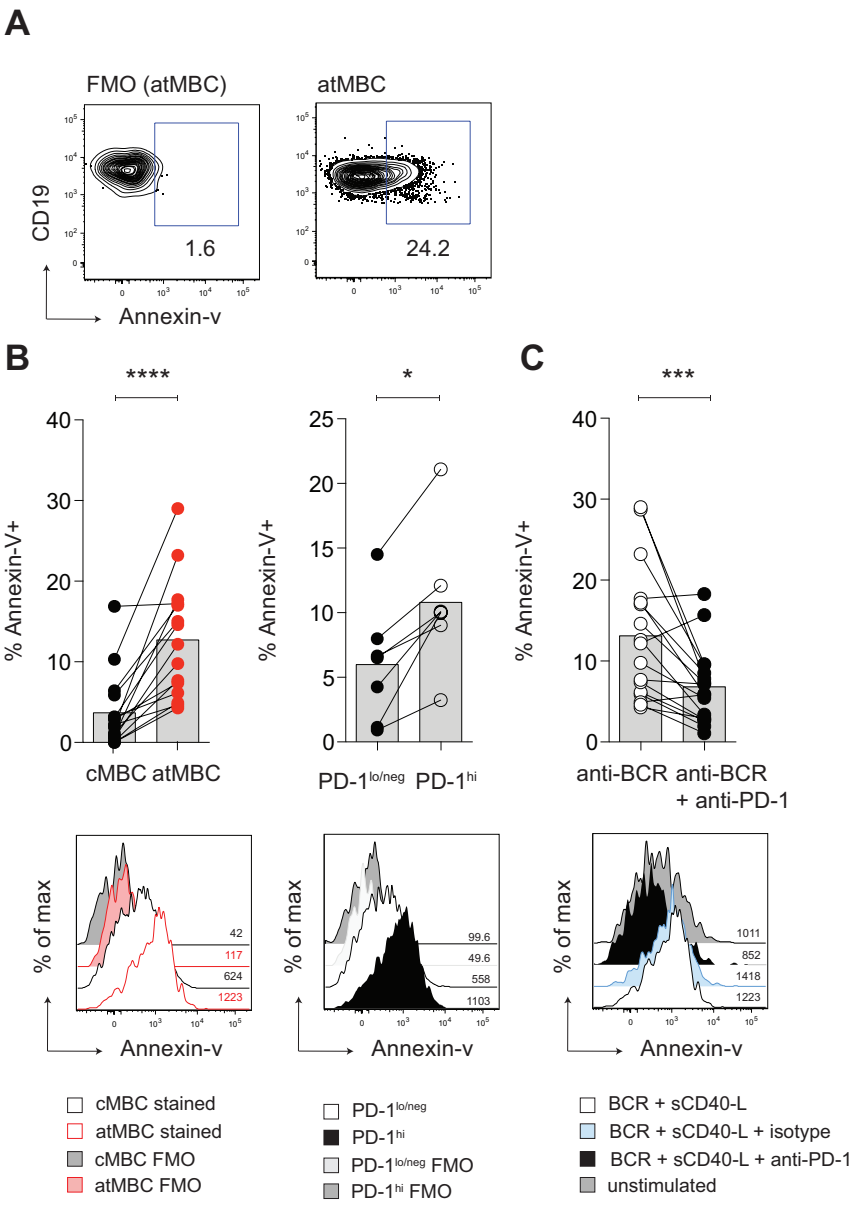

Figure 4-19 Expression of Annexin-V on atMBCs following *in vitro* stimulation

A. Representative staining of Annexin-V on atMBCs after stimulation with F(ab')$_2$ anti-IgG and -IgM (1ug/ml) and CD40-L (0.5ug/ml) for 4 d. **B.** Percentage of cells expressing Annexin-V stratified by cMBC and atMBC phenotype (n=15) and by PD-1 expression on total B cells (n=7 patients with CHB), following BCR stimulation and CD40-ligation. **C.** Annexin-V expression on atMBCs stimulated ± anti-PD-1 mAb or matched isotype (clone EH12.2H7; 10ug/ml) for 4 d (n=17). Error bars indicate mean ± SEM; *, P < 0.05; ***, P < 0.001; ****, P < 0.000; p-values were determined by Wilcoxon signed-rank test (b-c).

4.3.10 Antiviral function of atMBCs is partially rescued by *in vitro* PD-1 blockade

Data collected thus far suggests that antigen-experienced atMBCs have upregulated expression of inhibitory receptors, which may in turn be responsible for repressing their function and/or promoting premature apoptosis. To further elucidate mechanisms of suppression at play, and to test putative immunotherapeutic targets for rescue of antiviral potential, we examined the effect of *in vitro* blockade of inhibitory receptors and assessed recovery of function by cytokine production.

Blockade of PD-1 ligands, PD-L1 and PD-L2, has previously been shown by our group and others to rescue cytokine production in HBV-specific T cells in CHB (Boni et al., 2007; Schurich et al., 2013). Initial evidence showed that PD-L1/2 blockade had minimal restorative effects on cytokine production by atMBCs stimulated overnight (data not shown). However, this could have been due to partially stimulatory effect of the PD-L1 mAb on B cells expressing this ligand. Therefore, we next tried blocking PD-1 receptor directly, as in previous reports. PD-1 blockade of B cells from patients with HIV improved responses to viral antigen associated with in increased expression of activation markers on treated cells (Nicholas et al., 2013). Similarly, *in vivo* PD-1 blockade in SIV-infected macaques enhanced antibody responses to non-SIV and SIV antigens, providing evidence for PD-1 mediated repression of humoral responses (Titanji et al., 2010).

In vitro PD-1 blockade resulted in modest increases in the proportion of atMBC able to produce IL-6 after short-term BCR cross-linking, greater than that observed by the addition of an isotype control. Consistent with their lower expression of PD-1, cMBC did not increase IL-6 production upon PD-1 blockade (Figure 4.20). Combined, these data suggest that PD-1 may be partially responsible for restraining the antiviral function of atMBCs, and hint at the capacity for inhibitory receptor blockade to restore atMBC function.

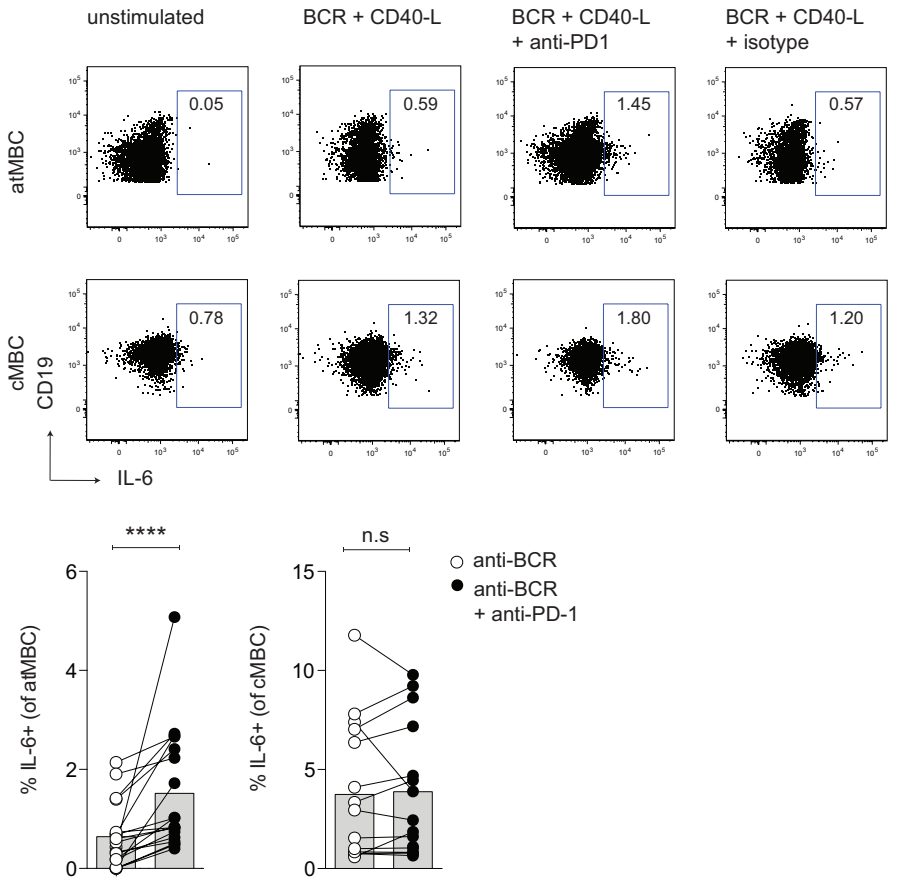

Figure 4-20 IL-6 production by atMBCs following PD-1 blockade

Intracellular staining for IL-6 on atMBCs and cMBCs, stimulated with F(ab')$_2$ anti-IgG and -IgM (1ug/ml) and CD40-L (0.5ug/ml) ± anti-PD-1 or matched isotype mAb for 16-18hrs (n=18). Frequency is presented minus a paired unstimulated control. Error bars indicate mean ± SEM; ****, P < 0.0001; p-values were determined by Wilcoxon signed-rank test (a-c).

4.3.11 Preliminary evidence showing partial rescue of antibody production in HBsAg-specific B cells by PD-1 blockade

Accordingly, we were next interested in examining the effect of PD-1 blockade on the capacity of B cells to differentiate to anti-HBs secreting plasma cells, since reduced *in vitro* production of anti-HBs was demonstrated in B cells from patients with CHB (Chapter Three). Global B cells were magnetically enriched from PBMCs isolated from patients with CHB and stimulated as before in the presence of anti-PD-1 blocking mAb. Again, comparison to IgG-secretion, as measured by ELISPOT, confirmed that viable cells capable of producing antibodies were formed and seeded. PD-1 blockade increased the frequency of anti-HBs secreting cells in 4/7 patients tested; however, responses still remained minimal (Figure 4.21A).

Hence, we hypothesised that additional signals may be required to rescue antibody production by HBsAg-specific cells. Therefore, we cultured B cells in association with an irradiated CD40-ligand

expressing CHO cell line as a surrogate for T cell help. This was performed in seven patients with CHB with data shown for the four patients who demonstrated anti-HBs production in any condition. Three patients failed to produce detectable anti-HBs responses in either condition, despite showing positive total IgG responses, and are not shown. Comparison to cells cultured in the presence of CD40-negative cells (performed where there were sufficient numbers of cells) showed that co-stimulation via CD40-ligand marginally increased anti-HBs responses in 2/5 patients, irrespective of PD-1 blockade (patients three and four; Figure 4.21B, C).

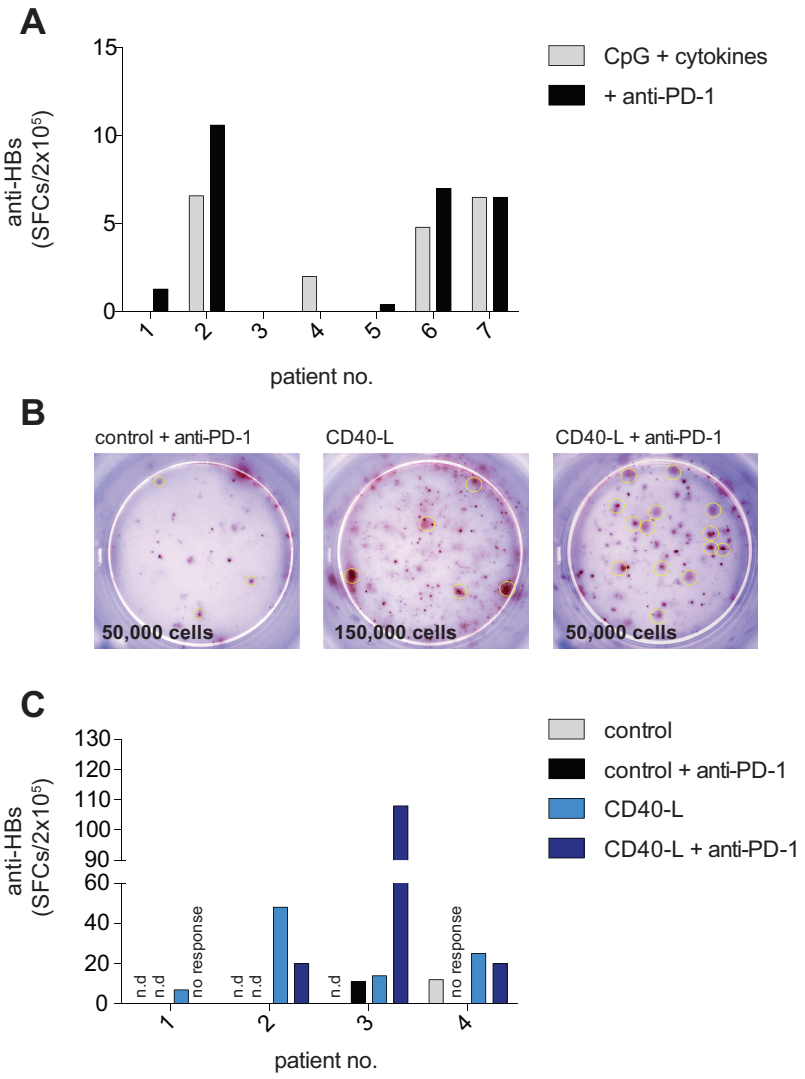

Figure 4-21 Effect of CD40 co-stimulation and PD-1 blockade on anti-HBs production

A. Frequency of anti-HBs producing cells following polyclonal stimulation of purified global B cells ± PD-1 blockade, determined by ELISPOT. **B-C.** Representative ELISPOT-well images (B) and cumulative data (C) showing anti-HBs producing cells in B cells stimulated ± CD40-L-expressing feeder layer and ± PD-1 blockade. Experiment was performed in 7 patients with CHB; only those that had detectable results in any condition are shown. SFC; Spot Forming Cells. All ELISPOTS were performed using purified global B cells. Each assay was performed in triplicate and normalised according to the number of cells seeded (denoted in bold).

CD40 stimulation in the presence of *in vitro* PD-1 blockade had variable effects; some patients (e.g. patient two) showed a minimal decline in anti-HBs secretion following PD-1 blockade, whilst patient five showed a significant increase in the number of spot forming cells, suggestive of a partial recovery of anti-HBs production (Figure 4.21B, C). Overall, the effects of PD-1 blockade showed inconsistent responses; much more work on a larger cohort of patients is required to determine its efficacy. It is likely that additional mechanisms also contribute to restraining HBsAg-specific B cells.

4.3.12 Preliminary evidence for a role of immune complexes in regulating atMBC/B cell responses

The identification of inhibitory Fc receptors on atMBCs and HBsAg-specific B cells may also have important roles in the regulation of B cell responses in CHB. Recent work has demonstrated that FcRL5 binds intact IgG (Franco et al., 2013; Wilson et al., 2012), suppressing B cell activation in a similar way to the inhibitory Fc receptor, FcγRIIB (Karnell et al., 2014; Smith and Clatworthy, 2010; Szili et al., 2014). Physiologically, these mechanisms likely have a role in tuning down antibody production by activated B cells once sufficient levels of immunoglobulin are detected. However, high expression of these inhibitory receptors in atMBCs raises the possibility that circulating immune complexes may crosslink Fc receptors and the BCR, resulting in suppression of BCR signalling. Moreover, expression of FcRL5 is thought to be induced upon sustained antigenic stimulation of the BCR (Damdinsuren et al., 2016). In this way, crosslinking of FcRL5, in combination with co-stimulation of BCR and TLR-9, results in a transient increase in FcRL5 expression, and can drive the differentiation of naïve B cells to antigen-primed, switched B cells (Dement-Brown et al., 2012). Therefore, we were next interested in examining whether circulating HBsAg:anti-HBs immune complexes, a well-recognised feature of CHB, further contribute to the suppression of B cells in HBV (Gerlich, 2007).

To begin to investigate this, we first isolated and cloned two anti-HBs antibodies – referred to as E6 and C7 - from a vaccinated healthy control, and generated immune complexes through incubation with recombinant HBsAg for two hours at 37°C (cloning was performed by Dr. Laura McCoy, Division of Infection and Immunity, UCL). Synthesised immune complexes underwent initial confirmation using size-exclusion chromatography to determine their relative size to individual, un-complexed components, with inconclusive results (collaboration with Prof. Mark Cragg and Dr. Richard Stopforth, University of Southampton). Supposed complexes made using C7 IgG ("C7 complex") eluted at a similar point to HBsAg alone, possibly indicative of a failure to incorporate IgG or formation of smaller antibody-antigen complexes (Zhang et al., 2015). E6 complexes were of a size more typical of immune complexes, as shown by comparison to a

reference immune complex. Both E6 and C7 IgG alone eluted later, indicative of their smaller size; however, they appeared much smaller than expected for unknown reasons (Figure 4.22A).

Preliminary testing for a possible inhibitory effect on BCR signalling, was examined by the phosphorylation of BLNK (p-BLNK) after short stimulation with $F(ab')_2$ anti-IgM/IgG/IgA-specific antibodies in the presence or absence of immune complexes. Cells stimulated in the presence of "C7 complex" showed suppressed phosphorylation in both total B cells (Figure 4.22B) and atMBCs (Figure 4.22C), shown by a shift in MFI compared to anti-IgM/G/A stimulation alone. Further analysis showed that stimulation in the presence of both C7 and E6 IgG alone suppressed B cells to a similar extent (Figure 4.22D, E), and at comparable levels to the C7 complexes. However, the size-exclusion chromatography data indicated that C7 complexes did not represent IgG alone, suggesting that there may be some aggregation of anti-HBs IgG to HBsAg, that is able to exert an inhibitory effect on B cell activation. Alternatively, decreased phosphorylation in the presence of C7 complexes may be simply explained by sequestration of HBsAg by IgG, thereby prohibiting stimulation of B cells via HBsAg. Complexes consisting of E6 IgG had a stimulatory effect compared to anti-IgM/G/A stimulation alone, comparable to the effect observed with HBsAg alone (Figure 4.22E). One interpretation may be that E6 complexes consist only of HBsAg, thus explaining the similarity to HBsAg alone, whereas C7 complexes represent true aggregates.

Immune complexes were also tested using a luciferase assay for the detection of IC-mediated SHP-1 recruitment to FcγRIIB (Stopforth et al., 2018) (Figure 4.22F). C7 "immune complexes" consisting of various ratios of C7 IgG to HBsAg were tested in comparison to PBS alone and a control immune complex. A 1 : 1 ratio of HBsAg to C7 IgG increased fluorescence above that of PBS alone and monomeric IgG, indicative of low-level binding to FcγRIIB (data courtesy of Dr. Richard Stopforth) (Figure 4.22F). However, this was significantly lower than that demonstrated by the control immune complex and not sufficient to confirm immune complex formation; further optimisation is thus required create definitive anti-HBs:HBsAg and test their effect on atMBCs. Finally, the suppressive effects observed using supposed immune complexes may be reflective of sequestration of HBsAg by IgG, therefore negating its stimulatory effect. Future studies should aim to test the effect of patient-isolated complexes on Ca^{2+} mobilisation and phosphorylation of BCR signalling components, and the subsequent impact of immune complex-mediated suppression on antiviral effector function. A recent paper lends support to this hypothesis, showing a role for IgG3 in dampening BCR responses via FcγRIIB (Kardava et al., 2018). Moreover, secreted IgG3 was found bound to IgM-expressing B cells *in vivo* in HIV-infected, chronically viraemic individuals, revealing a new way in which antibodies are able to regulate humoral responses.

One important consideration is that HBsAg incubated at this concentration alone may form multimers and exert some inhibitory effect itself (Siegler and Bruss, 2013). Therefore, we compared

HBsAg incubated for two hours at 37ºC with untouched HBsAg. When considering the percentage of cells expressing p-BLNK, "complexed" HBsAg was more suppressive than "uncomplexed", in line with this prediction (Figure 4.22D, E).

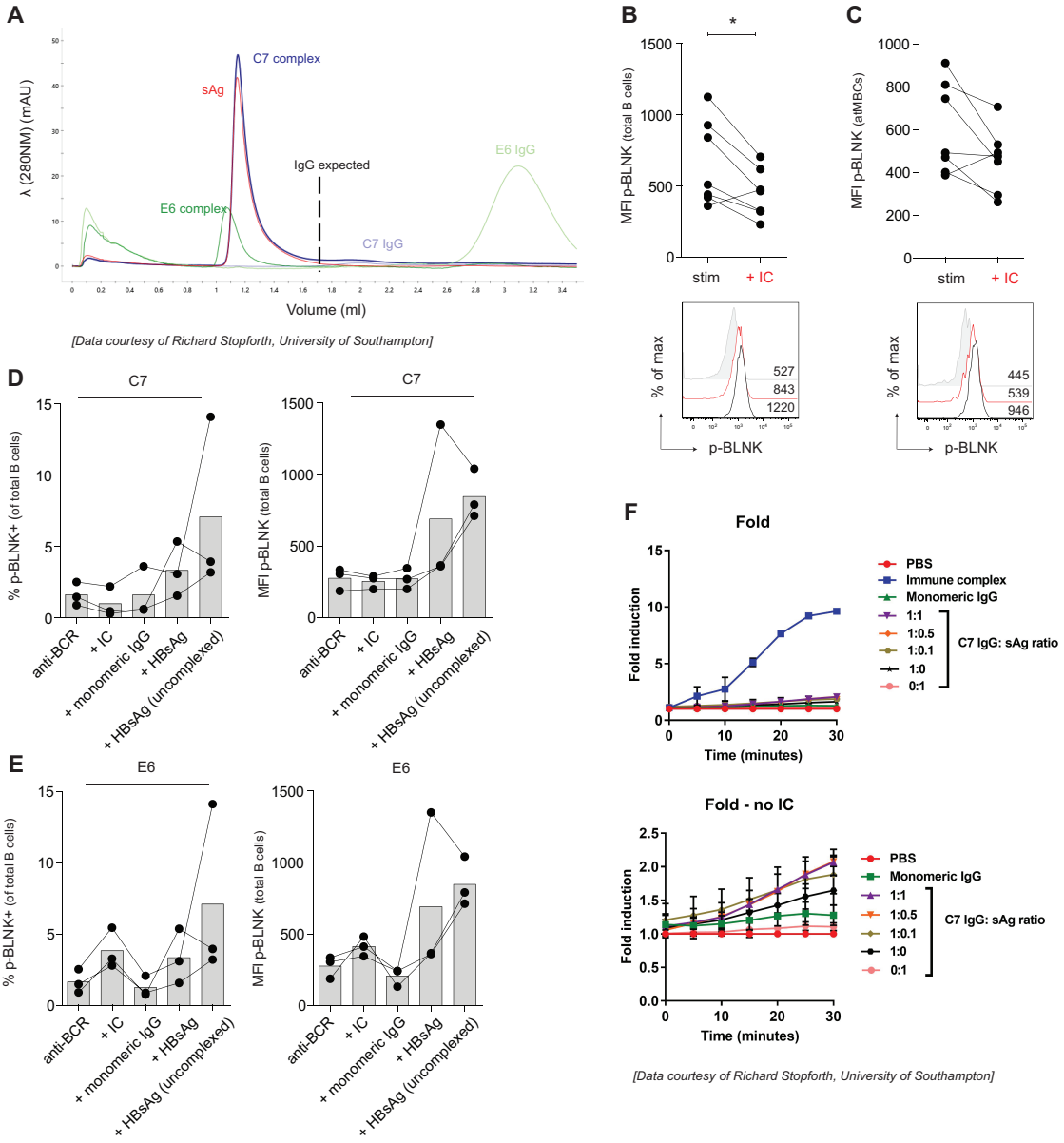

Figure 4-22 Preliminary data investigating a role for immune complexes in suppressing atMBC function

A. HBsAg-specific B cells were isolated via single-cell FACS-sorting and the κ and heavy chains sequenced (experiments performed by Dr. Laura McCoy). 293T cells were transfected and cultured for five days at 37°C. Antibody-containing supernatant was collected and total IgG isolated using Protein G beads. Purified antibody was concentrated and immune complexes (IC) were made through incubation of 100 µg/ml of C7 or E6 anti-HBs IgG with 100 µg of HBsAg for 2 h at 37ºC. Formation was assessed using size-exclusion chromatography and compared to C7 and E6 IgG alone and HBsAg alone (data courtesy of Dr. Richard Stopforth, University of Southampton). **B-C.** Representative example and summary data: phosphorylation of BLNK (p-BLNK; MFI) after stimulation with $F(ab')_2$ anti-IgM/A/G (20 µg/ml) in the presence or absence of IC in total B cells (**B**) and atMBCs (**C**) in patients with CHB (n=7). **D-E.** Phosphorylation of BLNK on total B cells (MFI and %) after stimulation with $F(ab')_2$ anti-IgM/A/G (20 µg/ml) in the presence or absence of IC, monomeric IgG alone or HBsAg alone (C7, **D;** E6, **E**). Untouched HBsAg was used for comparison. Solid lines connect paired samples from within the same individual (n=3). **F.** Comparison of IC binding to FcγRIIB determined by a luciferase assay, with and without a control IC, for scale (data courtesy of Dr. Richard Stopforth, University of Southampton). Error bars show mean ± S.E.M; *, $P < 0.05$; p-values were determined via Wilcoxon signed-rank test (b and c).

4.4 Discussion

Ours is the first study to phenotypically analyse antigen-specific B cells in patients with CHB. Data in previous chapters revealed that HBsAg-specific B cells are present in the circulation of patients with CHB but show impaired ability to secrete anti-HBs. Here, we compared the phenotype of antiviral B cells in patients with CHB to those induced by HBV-vaccination. We show that HBsAg-specific classical $CD27^{+}$ memory B cells are depleted in the context of CHB infection relative to vaccine-induced immunity. In contrast, HBsAg-specific B cells in CHB, and to a lesser extent the global B cell compartment, are enriched for B cells with a T-bet^{hi} atypical phenotype, characterised by low or negative expression of CD27 and CD21. Through characterising global atypical memory B cells (atMBCs), we show that these cells have upregulated expression of a number of inhibitory receptors, particularly PD-1 and FcR-like receptor 5 (FcRL5). Accordingly, atMBCs demonstrated compromised antiviral function, characterised by attenuated Ca^{2+} mobilisation and impaired cytokine production in response to BCR triggering, and a diminished ability to differentiate into antibody-producing plasma cells. *In vitro* blockade of PD-1 suggested that dysfunction may be partially attributable to the increased levels of inhibitory receptors described in atMBCs, offering a potential target for enhancing B cell immunity in patients with CHB. However, further analysis is required to ascertain the mechanisms by which these cells are functionally restrained. Overall, we postulate that an expansion of PD-1^{hi} global and HBsAg-specific atMBCs may result in impaired B cell-mediated antiviral immunity in patients with CHB. These findings are summarised in Figure 4.23.

$CD27^{-}CD21^{low/-}$ B cells have been described in various settings, providing clues to their roles in human disease. Accumulating evidence suggests that these cells arise following persistent microbial challenge, but also accrue as a result of ageing and in patients with autoimmunity. Combined, these data point to an expansion of atMBCs driven by persistent antigenic stimulation, whether by pathogenic- or self-antigen. In line with these reports, the accumulation of these cells in CHB appears to be, at least in part, driven by chronic exposure to HBV-viral proteins, as evidenced by the higher frequency of atMBCs in patients with ongoing infection, compared to healthy controls. Due to the high circulating levels of HBsAg in patients with CHB, it follows that atMBCs were enriched within the HBsAg-specific fraction during acute and chronic infection, and decreased following resolution of disease.

We propose that the expansion of $CD21^{low}$ B cells may interfere with the formation of functional humoral immune responses in patients with CHB, whereby the normal development of memory B cells is subverted towards a dysfunctional phenotype. In contrast to autoimmune conditions, which describe them as a naïve-like, non-memory population, $CD27^{-}CD21^{low/-}$ atMBCs cells have been carefully delineated as memory B cells in models of chronic infection and ageing

(Thorarinsdottir et al., 2016). Deep sequencing of V_H regions suggested that atMBCs differentiate from cMBCs as a result of continued antigen exposure (Portugal et al., 2015). In these studies, atMBCs and cMBCs displayed comparable rates of somatic hypermutation and clonal expansion; analysis of cell divisions, via measurements of κ-deletion recombination excision circles, further confirmed that both cell types had undergone a similar number of cell divisions. These data were in agreement with studies of age-associated B cells, which showed a stochastic representation of variable light and heavy immunoglobulin genes, suggesting that they arise from within the full BCR repertoire (Knode et al., 2017). Clonality analysis of demonstrated evidence of a close clonal relationship between $CD27^+$ and $CD27^-$ memory B cells (Wu et al., 2011). Combined, these data imply that atMBCs and cMBCs share a common ancestry and undergo similar antigen-driven expansion in the germinal centre.

Activated, $CD27^+CD21^{low/-}$ memory B cells also represent a population of antigen-experienced B cells, but are supposedly intermediate of cMBCs and atMBCs in terms of the extent to which they have undergone affinity maturation (Moir et al., 2008; Obeng-Adjei et al., 2017). Therefore, the expansion of these cells, within the global and antigen-specific B cell response, may equally detract from the generation of long-lived, specific and protective humoral responses. Based on these reports, we propose that chronic exposure to HBsAg perturbs formation of memory B cells, leading to an accumulation of dysfunctional atMBCs. However, further investigation of the BCR repertoire and immunoglobulin gene usage in memory B cell subsets and HBsAg-specific B cells in CHB, would reveal the extent to which these cells are shaped by chronic HBsAg stimulation, and the pathways by which they arise.

Antiviral effector functions of memory B cells, such as antibody and cytokine production, are induced upon secondary encounter of antigen by the BCR. Binding of antigen triggers complex signalling events that not only control the proliferation of the cell, but also determine downstream differentiation and function. In this regard, $CD27^-CD21^{low/-}$ B cells have been described as hypo-responsive to BCR stimulation, demonstrated by diminished proliferation and immunoglobulin secretion following BCR crosslinking (Charles et al., 2008; Isnardi et al., 2010; Portugal et al., 2015; Rakhmanov et al., 2009). We showed that *in vitro* stimulation of the BCR resulted in poor Ca^{2+} mobilisation in atMBCs relative to $CD27^+$ memory B cells, and an impaired ability to secrete antiviral cytokines.

The observed defect in BCR signalling and Ca^{2+} flux may have important implications in the resulting effector function of atMBCs. Ca^{2+} mobilisation is involved in the regulation of transcription factor expression, including NFAT and NFκB, that in turn control cell activation and differentiation (Hogan et al., 2003; Scharenberg et al., 2007). The reduced Ca^{2+} mobilisation demonstrated by atMBCs may be the reflective of differences in immunoglobulin isotype

expression, as IgG^+ B cells have been shown to have amplified responses to BCR triggering relative to naïve or IgM^+ memory B cells (Davey and Pierce, 2012). However, *ex vivo* analysis suggested that a higher proportion of atMBCs were class-switched relative to naïve B cells, yet both subsets displayed comparable levels of Ca^{2+} mobilisation following BCR stimulation.

Further analysis of signalling pathways downstream of the BCR could help pinpoint defects in atMBC responses in patients with CHB. Analysis of activation levels of Syk may be particularly informative, as Syk has been shown to be the key signal transducer required for coupling the BCR to downstream signalling pathways in mature B cells. Ackermann et al., showed that the presence of Syk is required for the activation and class-switching of B cells in response to BCR stimulation and CD40-ligand stimulation respectively. They demonstrated that Syk-deficient B cells fail to differentiate into germinal centre or plasma B cells, and have defective antibody responses following T-dependent stimulation (Ackermann et al., 2015). Thus, defects in the activation of Syk in atMBCs may account for many of the failings in antiviral function observed.

Decreased Ca^{2+} flux was mirrored by an impaired ability to secrete cytokines following BCR crosslinking, indicative of impaired antiviral function of atMBCs in patients with CHB following antigen stimulation. This defect may have important consequences for the antiviral control of HBV. TNFα, together with IFNγ, plays a central role in the non-cytolytic control of HBV, through the inhibition of virus replication and degradation of cccDNA in infected cells (Guidotti et al., 1996; Xia et al., 2016). IL-6 has also been linked to cytokine-mediated inhibition of HBV, through modes of action such as the downregulation of the entry receptor on host cells (Bouezzedine et al., 2015), and the disruption of viral transcription and cccDNA acetylation (Hösel et al., 2009; Palumbo et al., 2015). The maturation of B cells into immunoglobulin producing cells is also IL-6 dependent (Muraguchi et al., 1988), with IL-6 shown to promote the generation of T_{FH} cells and assist viral control in latter stages of infection (Harker et al., 2011). Our documentation of the aberrant production of IL-6 and TNFα by atMBC in CHB, therefore strongly implicates atMBCs in limiting antiviral responses.

Of note, previous reports have described that whilst hypo-responsive to BCR stimulation, atMBCs can maintain responses to TLR stimuli. Our data indicated that both plasma cell differentiation and cytokine stimulation were significantly decreased in atMBCs relative to cMBCs, even when stimulated through TLR-ligands. It is possible that this is reflective of the nature of chronic stimulation occurring in CHB. Circulating antigen-specific B cells are exposed to high levels of TLR stimuli in the liver, in the form of microbial products draining from the gut (Henao-Mejia et al., 2013). Moreover, CPG motifs have been identified in HBV viral particles, with HBsAg also shown to trigger TLR-9 (Tout et al., 2018). Therefore, in a similar manner to chronic antigen stimulation impairing responses to BCR signalling, persistent stimulation through TLR may result in a tolerisation of atMBCs to TLR triggering in patients with CHB. Overall, we postulate that atMBCs

require a higher threshold of activation than cMBCs, and may necessitate multiple stimuli prior to activation and differentiation.

Decreased expression of CD21 may be partly responsible for both attenuating BCR signalling and effector function, as CD21, in the context of the BCR co-receptor, contributes to decreasing the threshold of stimulation required for B cell activation. However, additional defects are likely imparted by inhibitory receptors expressed by atMBCs and HBsAg-specific B cells. We postulate that this attenuation of BCR signalling, in combination with a loss of adaptive immune cell function, may be reflective of the upregulated expression of inhibitory receptors, including FcRL5 and PD-1, as both are able to modulate B cell receptor signalling and downstream function. In mice, gain of FcRL5 expression is associated with a switch from adaptive to innate-like function (Li et al., 2014), with FcRL5 shown to exert binary, compartment-specific effects on BCR function in innate-like marginal zone B cells (Zhu et al., 2013). Comparatively, in human memory B cells, FcRL5 inhibits B cell activation by recruiting phosphatases SHP-1/2 to ITIM motifs in the B cell receptor, thereby inhibiting Ca^{2+} mobilisation and downstream signalling (Haga et al., 2007). Of note, FcRL5 demonstrates heterogeneous expression on atMBCs in CHB and on dysfunctional B cells from other chronic infections. Previous studies have shown that FcRL5^{+} cells have significantly deficient function compared to FcRL5^{-} B cells (Li et al., 2016; Sullivan et al., 2015). These studies showed that FcRL5^{+} B cells had a decreased capacity to differentiate into ASCs and were more prone to apoptosis when cultured. Moreover, studies demonstrating defective phosphorylation of BCR signalling components, Syk, Plc-γ2 and BLNK, in CD27^{-}CD21$^{low/-}$ relative to CD27^{+} memory B cells, demonstrated attenuated signalling associated with increased expression of FcRL5 (Portugal et al., 2015).Therefore, it is likely that the FcRL5^{+} atypical memory B cells represent the most dysfunctional population.

B cell expression of PD-1 is also associated with aberrant activation of B cells and resultant loss of function in the context of HIV and SIV infection (Nicholas et al., 2013; Titanji et al., 2010). Co-ligation of PD-1 and the BCR inhibited Ca^{2+} mobilisation and tyrosine phosphorylation of BCR signalling molecules, including Syk and Plc-γ2, in a manner similar to FcRL5 (Okazaki et al., 2001). Altering expression of these inhibitory receptors could therefore regulate the B cell response to antigen. A paper from Kardava et al., used siRNA knockdown to downregulate expression of nine inhibitory receptors preferentially expressed on CD27^{-}CD21$^{low/-}$ B cells, including PD-1 and FcRL4 – a closely related molecule to FcRL5 shown to be highly expressed on CD27^{-}CD21$^{low/-}$ B cells in HIV. Downregulation of these molecules significantly increased BCR-induced proliferation and cytokine secretion (Kardava et al., 2011), suggesting that it may be possible to rescue the function of HBsAg-specific B cells in CHB through the repression of inhibitory receptors.

Consistent with this, we were able to modestly enhance the capacity of atMCBs from patients with CHB to secrete IL-6 via PD-1 blockade following BCR stimulation. *In vivo* PD-1 blockade in macaques infected with simian immunodeficiency virus (SIV) demonstrated enhanced proliferation of memory B cells and production of SIV-envelope specific antibodies, concurrent with expansion of virus-specific T cells (Velu et al., 2009). This may have had indirect effects via boosting of T_{FH} *in vivo*; however, previous work in mice has demonstrated a PD-1 blockade dependent inhibition of T_{FH} interactions in germinal centres (Good-Jacobson et al., 2010). The direct effects of PD-1 on B cells has been further demonstrated, again in SIV-infected macaques, showing preferential depletion of PD-1 expressing memory B cells *in vivo,* and an increased susceptibility to PD-L1-dependent apoptosis in PD-$1^{hi}$$CD21^{-}$ actMBCs *in vitro* (Titanji et al., 2010). This latter finding in particular supports our data showing a 3-fold decrease in apoptosis upon PD-1 blockade.

In contrast, we were not able to detect significant recovery of anti-HBs responses upon PD-1 blockade and CD40 co-stimulation, despite a back-to-back publication showing rescue of anti-HBs production in four patients with CHB (Salimzadeh et al., 2018). PD-1 blockade has shown marked variation in success between individuals, with *in vitro* assays reporting increased effector function of HBV-specific T cells in ~30-50% of patients sampled (Schurich et al., 2013). Therefore, it is possible that improved responses may be observed once expanded to a wider cohort of individuals. It is also possible that rescue of B cell effector function is counter-balanced by inhibition through Fc receptors, induced by binding of the Fc portion of monoclonal antibodies to inhibitory receptors on the B cell. Therefore, it is likely that additional signals, for example boosting of co-stimulatory T_{FH} function or blockade of synergistic inhibitory receptors, are required to optimise B cell recovery.

Our data showed close association of PD-1 with the expression of the transcription factor T-bet, as also demonstrated in malaria, HCV and HIV (Chang et al., 2017; Knox et al., 2017; Obeng-Adjei et al., 2017; Portugal et al., 2015). T-bet expression was elevated in atMBCs compared to cMBCs, and as such, was significantly enriched in HBsAg-specific B cells from patients with CHB relative to vaccinated healthy controls. This finding is in stark contrast to data suggesting that induction of T-bet in virus-specific CD8 T cells represses PD-1 expression, and thus correlated with the functional recovery of exhausted T cells (Kao et al., 2011; Kurktschiev et al., 2014; Schurich et al., 2013). Contrary to our findings, evidence emerging from murine models postulates that T-bet expression in B cells has a positive role in promoting viral clearance (Barnett et al., 2016), as outlined earlier. Moreover, findings from acute yellow fever and vaccinia vaccination demonstrated infection-induced activation of T-$bet^{hi}$$CD27^{-}$$CD21^{low/-}$ B cells, suggesting that they actively participate in antiviral immunity (Knox et al., 2017). One interpretation may be that T-bet^{+} B cells may contribute to acute antiviral response, yet become dysfunctional when they are maintained for extended periods of time, such as in CHB. Alternatively, T-bet^{hi} B cells may represent functional, antigen-experienced B cells, however, function is restrained when expression coincides with

exhaustion-induced inhibitory receptor expression. This concept is supported by a recent study which showed that T-bet expression was associated with expression of the co-inhibitory molecule, FcγRIIB and inversely correlated with the efficiency of BCR signalling (Obeng-Adjei et al., 2017). These paradoxical findings are reminiscent of the complex role of PD-1 on T cells, associated with both activation and inhibition, limiting T cell function whilst also promoting long-term maintenance of immune-surveillance in the setting of chronic antigenic stimulation (Pallett et al., 2017; Wherry and Kurachi, 2015; Zehn et al., 2016). As a result, T-bet^{hi}PD-1^{hi}HBsAg-specific B cells may represent a favourable target to release from inhibition since they should be transcriptionally wired for antiviral efficacy. Further analysis of BCR signalling and antiviral function of PD-1-expressing B cells isolated *ex vivo*, as well as extension of data using anti-PD-1 blockade, would provide definitive evidence on the role of PD-1 in B cells.

The expansion of atMBCs and HBsAg-specific B cells expressing high levels of inhibitory receptors appears to be driven by chronic antigen stimulation. Analyses presented here indicated that the expression of inhibitory receptors on global atMBCs increases in accordance with active disease; PD-1^{hi} atMBCS were highest in the patients with viral loads above 2000IU/ml, compared to those with low levels of HBV DNA and healthy controls. This finding is similar to data showing that virus-specific T cells have high levels of PD-1 expression occurring as a result of chronic antigenic-stimulation (Boni et al., 2007). This interpretation is further supported by evidence that B cells expressing FcRL5, often co-expressed with PD-1, exhibit higher rates of somatic hypermutation and a more switched immunoglobulin profile than FcRL5^{-} B cells, despite showing reduced function (Li et al., 2016).

Building on this concept, our data suggested that FcRL5^{+} atMBCs may consist of two distinct subsets: one that has encountered antigen and undergone class-switching, and a second subset that has features of a more naïve cell type, characterised by expression of IgM. This theory is supported by data from a recent study which purported to the existence of naïve-like age-associated B cells in addition to antigen-induced age-associated B cells (Swain et al., 2017). The naïve-like B cells described expressed high levels of soluble IgM and were negative for expression of CD11c and CD80, in agreement with our data. In contrast, antigen-experienced age-associated B cells that increased during the course of murine influenza A infection, were mainly class-switched, and expressed higher levels of CD80/86, CD11c and CD38. Our data revealed a concentration of PD-1 expression on CD24^{-}CD38^{-}IgM^{-}IgD^{-} B cells, pointing to a population of antigen-experienced cells. Additional populations of FcRL5-negative CD24^{-}CD38^{+} and CD24^{+}CD38^{-} B cells, described as having similar functions to atMBCs, were also identified (Thorarinsdottir et al., 2016); however, their contribution to the CD27^{-}CD21$^{low/-}$ pool was minimal, constituting ~15% of the atMBC population.

Thus, it is increasingly thought that atMBCs may represent a heterogeneous pool of B cells, which have divergent and specialised functions. This heterogeneity is apparent not only within individuals, but seems also to exist between disease settings. Our observations, demonstrating the functional impairment of atMBCs to differentiate into ASCs, are in line with findings from *Plasmodium falciparum* infection, which showed that FACs-sorted atMBCs could not be stimulated to produce antibodies in conditions that otherwise promoted cMBCs to readily differentiate into ASCs (Portugal et al., 2015). This defect may be partially attributable to the expression of FcRL5, which was shown to suppress immunoglobulin production in FcRL5^{+} atMBCs and cMBCs (Sullivan et al., 2015). A contradictory report suggested that atMBCs are capable of producing neutralising antibodies to blood stage *Pf.* parasites, at a similar level to cMBCs. However, this study utilised measurements of immunoglobulin transcripts and therefore did not directly pertain to antibody production by B cells themselves (Muellenbeck et al., 2013). In HIV, analysis of affinity maturation revealed that rates of somatic hypermutation were significantly reduced in CD27^{-}CD21$^{low/-}$ B cells, relative to CD27^{+}CD21^{+} B cells, despite having undergone similar frequencies of cell divisions. Decreased levels of affinity maturation directly correlated with reduced HIV-neutralising capacity of the resultant antibodies, suggesting that the expansion of CD27^{-}CD21$^{low/-}$ B cells in HIV may contribute to impaired neutralisation of the virus (Meffre et al.; Moir et al., 2008).

In contrast, in settings of autoimmunity, immunoglobulin synthesis is enhanced in CD21^{low} B cells relative to CD21^{+} B cells, suggestive of preferential differentiation into antibody secreting cells (Rakhmanov et al., 2009). Notably, despite showing decreased levels of differentiation to ASCs, atMBCs from vaccinated healthy controls did not show reduced production of total IgG relative to cMBCs. This observation may be reflective of lower levels of inhibitory receptor expression identified on atMBCs from vaccinated healthy controls compared to patients with CHB. However, it could also be indicative of IgG production by CD21^{low} naïve-like B cells within the atMBC population alluded to previously. PD-1^{low} naïve-like atMBCs, described in this study, showed marked similarities to CD21^{low} B cells described in CVID that are thought to represent an anergic population responsible for the production of polyreactive antibodies (Isnardi et al., 2010). Therefore, it is possible that analysis of IgG production by atMBCs in healthy individuals is not representative of IgG production by atMBCs that are induced and impaired as a result of chronic exposure to HBsAg. Indeed, analysis of atMBCs isolated from one patient with CHB showed a marked reduction in IgG by atMBCs, compared to a matched number cMBCs; however, further analysis, currently limited by access to category three hazard sorting facilities, is needed to confirm these results. Future studies should now focus on assessing the quality of antibody produced by atMBCs in HBV, to examine whether this expansion of atMBCs within HBsAg-specific B cells directly contributes to the failure of patients with CHB to produce neutralising anti-HBs. Arguably the most definitive way to ascertain the relative contributions of these different subsets to HBsAg-specific immunity would be to analyse gene expression at the single cell level.

atMBCs also appeared to be more prone to apoptosis compared to cMBCs, as demonstrated by the increased levels of Annexin-V staining following BCR and CD40 stimulation, and the decreased survival and differentiation of atMBCs to ASCs. These data were supported by others showing that markers of apoptosis were enriched in $CD21^{low}$ B cells (Charles et al., 2008; Rakhmanov et al., 2009). Apoptosis of lymphocytes is central to the shaping the immune repertoire and terminating immune responses. However, the data presented here suggests that premature apoptosis may be responsible for curtailing the development of functional humoral immune responses in CHB. It is likely that reduced expression of CD21 plays a role in the survival of atMBCs, with CD21 shown to be necessary for the maintenance of B cells within the lymphoid compartment (Fischer et al., 1998). However, environmental signals may also play an important role in promoting premature apoptosis of atMBCs in patients with CHB. For example, persistent TLR-4 signalling triggered by microbial gut products in cirrhotic liver may predispose memory B cells towards FAS-mediated apoptosis (Chang et al., 2016). The susceptibility of atMBCs towards premature cell death is likely also a consequence of their dysfunctional phenotype. Upregulated expression of CD95 on $CD21^{low}$ B cells isolated from HIV-viraemic patients correlated with an increased propensity towards FAS-mediated apoptosis (Moir et al., 2004). $CD21^{low}$ B cells have also been reported to have decreased expression of IL-4R (Charles et al., 2008; Isnardi et al., 2010), which might otherwise confer resistance to IL-4-mediated protection against Bim-mediated apoptosis (Granato et al., 2014).

Data presented here may point to a role for PD-1 blockade in rescuing atMBCs from premature apoptosis. Although this may be beneficial in terms of preventing cell death of HBsAg-specific B cells, it raises the possibility that PD-1 blockade may actually potentiate the accumulation of dysfunctional cells within the memory B cell compartment. Pertinent to this concept, melanoma patients treated with combination CTLA-4 and PD-1 blockade showed an increased risk of developing immune-related adverse events, associated with the rapid appearance of circulating $CD21^{low}$ B cells expressing a non-lymphoid tissue-homing profile (Das et al., 2018). As a result, therapeutic blockade of inhibitory receptors may preferentially expand $CD21^{-}CD27^{low/-}$ B cells, in turn leading to adverse impacts on the generation of humoral immune responses and manifestations of autoimmunity (Naradikian et al., 2016b). This also highlights the importance of investigating bystander impacts to B cells of checkpoint blockade strategies aimed at rescuing T cell responses.

Clearly, the concentration of atMBCs with high inhibitory receptor expression within the HBsAg-specific compartment cannot wholly explain their defective anti-HBs production, since there remains a population of supposedly functional HBsAg-specific $CD27^{+}CD21^{+}$ cMBCs. Although diminished, it is possible that these cells do produce low levels of anti-HBs, accumulating to high enough levels for detection in some patients with comparatively high levels of HBsAg-specific

cMBCs. Interestingly, transcriptional analysis of naïve B cells and memory B cell subsets in patients with CHB compared to healthy controls, suggested that HBV-infection may also have significant impacts on supposedly functional B cell responses (Salimzadeh et al., 2018).

However, there are likely more generalised impacts of HBV infection on B cells that affect their ability to form protective humoral immune responses. One consideration is the profile of receptors that direct their localisation. Both atMBCs and the total HBsAg-specific B cell population expressed high levels of CD11c and CXCR3, alongside decreased expression of CXCR5. Expression of CXCR3 and CD11c may promote the migration of atMBCs and HBsAg-specific B cells to inflamed tissue sites (Xie et al., 2003). In autoimmunity, $CD11c^{+}CXCR3^{+}CD27^{-}CD21^{low/-}$ B cells have been shown to preferentially home to the bronchoalveolar space and synovial fluid in patients with CVID and rheumatoid arthritis, respectively (Rakhmanov et al., 2009). In opposition, CXCR5 expression facilitates migration of memory B cells to the B cell follicle, along a gradient of CXCL13 secreted by follicular dendritic cells resident in the lymphoid tissue. Taken together, we propose that altered expression of chemokine receptors may promote the migration of atMBCs and HBsAg-specific B cells to inflamed tissues and away from non-lymphoid sites, thereby reducing the opportunities for productive interactions with CD4 T helper cells and antigen-driven affinity maturation. Due to the centrality of CD4 T cell – B cell interactions to the development of high-affinity antibody, we postulate that impaired CD4 T cell help may contribute to the suboptimal antibody production observed in CHB. Furthermore, high levels of BCR stimulation in the absence of appropriate T cell help can lead to the induction of functional anergy and apoptosis in B cells; a fundamental mechanism underpinning the development of B cell tolerance.

In further evidence of this concept, we also observed decreased expression of CD40 on atMBCs relative to cMBCs, and on HBsAg-specific B cells from patients with CHB, compared to antigen-specific B cells isolated from vaccinated healthy controls. The interaction between CD40, expressed on B cells, with CD40-ligand on T_{FH} is central to the activation of B cells and the induction of class-switch recombination. Therefore, decreased expression of CD40 supports the concept that atMBCs and HBsAg-specific B cells experience impaired interactions with T helper cells, leading to diminished activation of B cells and decreased affinity antibody responses. This hypothesis may contribute to explaining the decreased proportion of class-switched B cells identified in global atMBCs. In addition, in models of ageing, an accumulation of $CD27^{-}CD21^{low/-}$ age-associated B cells was associated with expansion of 'aged' CD4 T cells that lead to decreased formation of germinal centres and long-lived plasma cell responses (Swain et al., 2017). The impact of this defect was an expansion of age-associated B cells that developed independently of T cell help, but required higher dose of PAMP-activation to produce antibodies. This finding illustrates how defective T cell help can result in hypo-responsiveness of memory B cells to stimulation, in line with our findings. Manipulation of the CD40 – CD40-L axis may therefore represent a promising therapeutic angle for rescuing both B cell – and T cell – mediated immunity in CHB.

In summary, atMBCs appear to represent a heterogeneous population of naïve-like and antigen-experienced B cells, the latter characterised by elevated expression of PD-1. These cells accumulate in the presence of HBsAg, showing increased frequency within the HBsAg-specific B cell fraction in patients with acute or chronic HBV, compared to HBV-vaccinated controls or HBV-resolved individuals. More work is required to ascertain whether the increase in atMBCs is a reflection of naïve or classical memory B cells becoming dysregulated, or a proliferation of naturally occurring atMBCs.

Investigation of global atMBCs revealed attenuated responses to BCR stimulation, demonstrated by decreased Ca^{2+} mobilisation, impaired cytokine production and susceptibility to cell death. Furthermore, atMBCs showed significant defects in their ability to differentiate into plasma cells, suggesting that their accumulation within HBsAg-specific responses may play a role in impairing humoral immunity in CHB. As a result of their decreased activation status, high expression of inhibitory receptors and impaired *in vitro* function, we propose that PD-1^+ atMBCs represent a functionally exhausted B cell subset, similar to exhausted T cells that accrue in chronic infection and cancer. Data presented here also supports a tentative role of PD-1 blockade in improving antiviral function and rescuing atMBCs from premature apoptosis. Further studies are necessary to test additional stimuli that bypass attenuated BCR signalling, and/or blocking synergistic inhibitory receptors, in order to optimise B cell recovery in CHB. The clinical application of such approaches will require caution to protect against the expansion of dysfunctional B cells with the potential to drive autoimmunity.

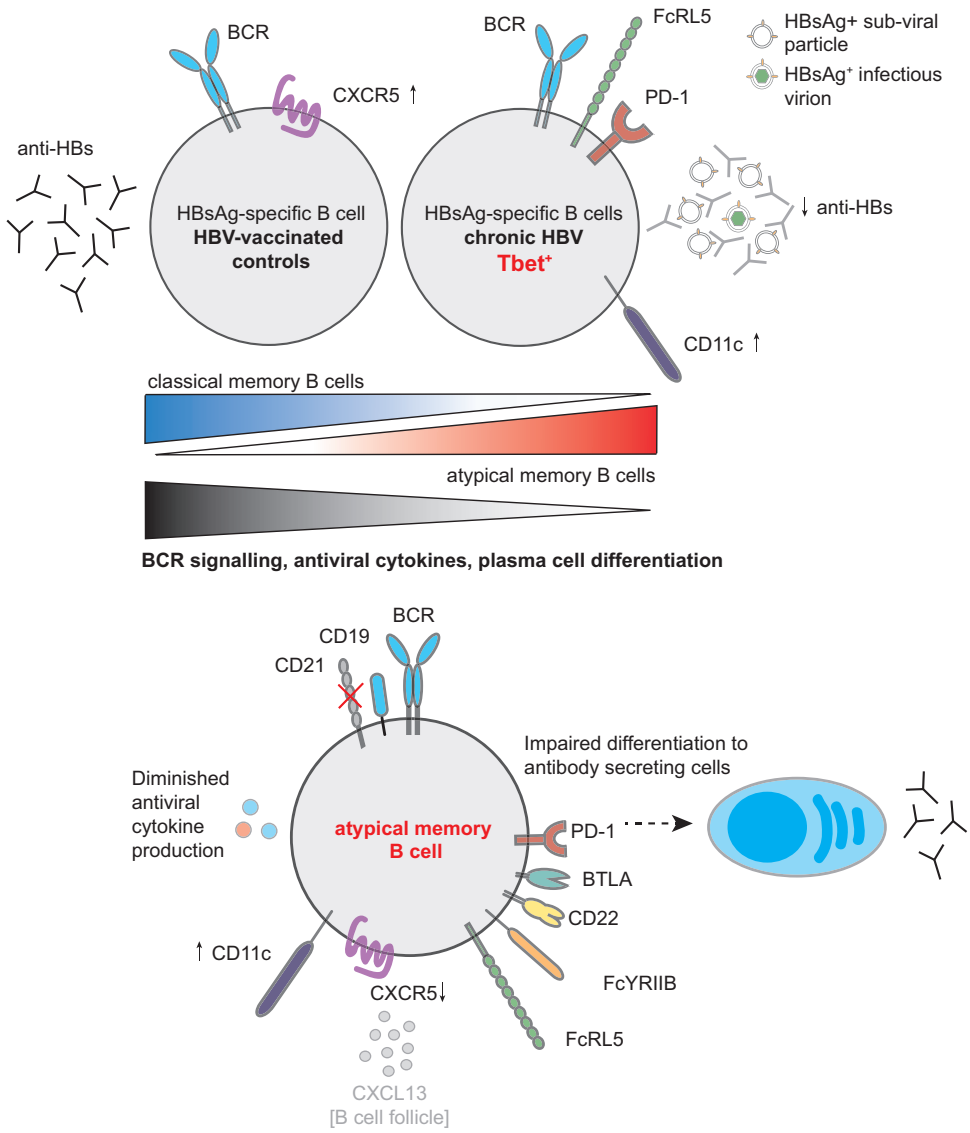

Figure 4-23 Summary of chapter findings

Summary of the phenotypic and functional changes described in HBsAg-specific B cells and atMBCs in patients with CHB

Chapter 5 HBsAg-specific B cells and atypical memory B cells in the liver

Chapter summary

It is increasingly appreciated that the phenotype, differentiation and function of immune cells is significantly impacted by the anatomical location in which they reside. Whilst many studies have identified important components of HBV disease pathogenesis through sampling the blood, it is now apparent that both the composition and function of lymphocytes is significantly altered in the liver (Gill et al., 2018) As an exclusively hepatotropic infection, it is therefore critical to understand how both the cellular composition of the liver and its microenvironment may regulate antiviral responses to infection. In this chapter, I examine the proportion of intrahepatic B cells with an atMBC phenotype and the persistence of HBsAg-specific B cells within the liver microenvironment.

5.1 Introduction

5.1.1 Immunology of the liver

Among very many roles (reviewed in (Michalopoulos, 2007)), the liver stands as the primary organ in the body responsible for the metabolism and detoxification of gut-derived products. These are delivered to the liver in the form of antigen-rich portal venous blood, which passes through a system of capillary-like vessels, referred to as sinusoids, before being metabolised by hepatocytes. As part of this, the liver also plays a key role in immune surveillance, whereby liver-specialised antigen-presenting cells scan the blood for invading pathogens. To do so, the liver must be uniquely tolerised to avoid immune responses against innocuous antigens that could lead to liver damage, whilst maintaining the ability to prime and elicit immune responses to blood-borne pathogens, where necessary (Crispe, 2009).

The ability of the liver to carry out immune surveillance is a function of both its anatomy and the diverse network of cells resident therein. Structurally, the liver is divided into hepatic lobules, which consist of a roughly hexagonal arrangement of hepatocytes that radiate out from the central vein (Figure 5.1). Blood enters the liver from the portal vein and flows through the sinusoids out into the systemic circulation via the central vein (Ishibashi et al., 2009). Hepatic sinusoids are lined by liver sinusoidal endothelial cells (LSECs), that can be distinguished by their large fenestrations that permit solute access to hepatocytes and accommodate membrane protrusions from both hepatocytes and infiltrating lymphocytes (Warren et al., 2006). Anatomically, LSECs separate hepatocytes from the blood flowing through the sinusoidal lumen, establishing formation of the Space of Disse between the endothelial lining and the underlying hepatocytes (Figure 5.1). The extensive narrow-lumen sinusoidal structure, combined with the low pressure of portal venous blood, results in a sluggish blood flow, and facilitates maximal interactions between circulating antigen and/or leukocytes and liver-resident antigen-presenting cells (Jenne and Kubes, 2013).

The tolerogenic nature of the liver was first alluded to following tolerance of fully MHC-mismatched pig liver transplants (Calne et al., 1969). This observation was later supported by the demonstration that co-transplantation of livers, alongside organs normally rejected alone, could promote tolerance of the donor organ (Benseler et al., 2007). Accordingly, liver transplant recipients have an increased propensity towards tolerance of the donor organ, and are able to tolerate withdrawal of immunosuppressive drugs with fewer consequences, in stark contrast to all other solid organ transplants (Benítez et al., 2013; Levitsky, 2013). Lastly, the liver is a common site for tumour metastases, again reflective of its tolerised nature.

Antigen-presentation in the liver is carried out by specialised liver resident cells, namely LSECs, Kupffer cells and hepatocytes. LSECs comprise ~50% of the non-parenchymal cells in the liver and play a critical role in the scavenging of pathogens and antigen presentation to T cells. This ability is conferred by the expression of a number of key cell-surface molecules, including pattern-

recognition receptors (TLR-3, TLR-4, TLR-7 and TLR-9), and the constitutive expression of MHC and co-stimulatory molecules (Jenne and Kubes, 2013). Through the expression of both MHC Class-I and MHC Class-II, LSECs can cross-present antigen to both CD4 and CD8 T cells (Schurich et al., 2009). Moreover, LSECs also express intracellular adhesion molecules 1 (ICAM-1), thereby facilitating their interaction with circulating leukocytes. Upon antigen presentation, LSECs upregulate expression of programmed death ligand 1 (PD-L1). In this way, LSECs also have a role in regulating lymphocyte responses; persistent engagement of PD-L1 with its receptor PD-1 on activated T cells culminates in the tolerisation of T cell responses (Berg et al., 2006).

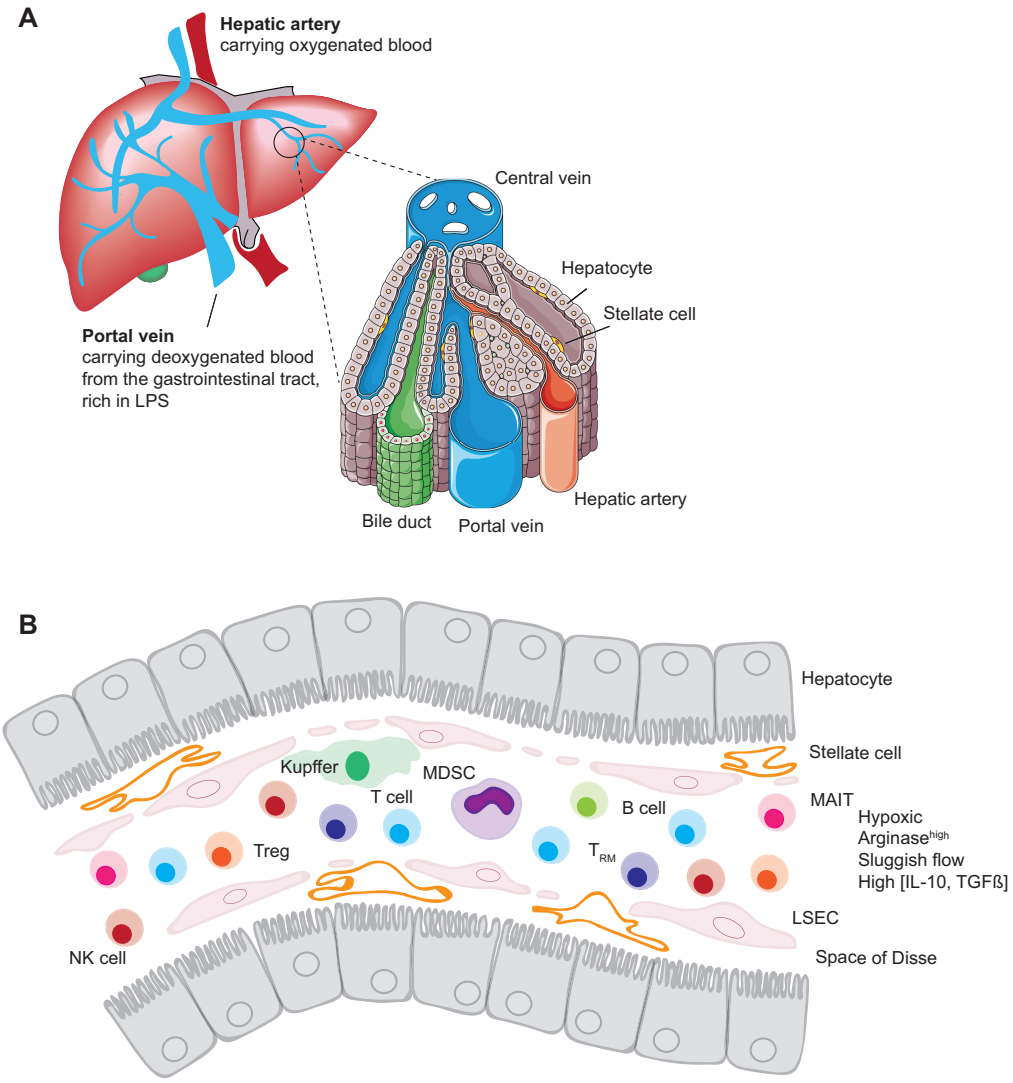

Figure 5-1 Schematic of the human liver microenvironment

Schematic of the human liver architecture and blood supply. **A.** A portion of a liver lobule. Blood flows from the portal vein and into the sinusoid, whereupon it is surveyed by liver-infiltrating and liver-resident lymphocyte populations. **B.** Detailed representation of the liver sinusoid. Hepatocytes are separated from the blood by non-parenchyma LSECs. The Space of Disse is defined as the space between the LSECs and the underlying hepatocytes, perforated by numerous fenestrate. Liver resident macrophages, Kupffer cells, adhere to the LSECs and rapidly clear pathogens from the blood. Adapted from (Maini and Pallett, 2018) and utilising Servier medical art (part A).

Kupffer cells (KCs) are a tissue-resident macrophage population that localise in the vasculature of the liver and express an array of scavenger receptors. These include TLRs, complement receptors, and Fc-receptors, that detect, bind and internalise pathogens. At rest, KCs predispose the liver towards tolerance through the secretion of immunosuppressive cytokines, IL-10 and TGFβ, and the expression of molecules, including Fas-ligand and PD-L1 (Boltjes et al., 2014). However, upon activation, KCs become potent antigen-presenting cells and produce a number of cytokines – namely, IL-12, IL-6 and TGFβ - that act to alert lymphocytes and amplify the immune response.

Hepatocytes represent the majority parenchymal cell type of the liver and have primary roles in the nutrient metabolism, protein production and toxin neutralisation. However, they also have additional roles in the adaptive immune response through the expression of MHC molecules: hepatocytes express high levels of MHC Class-I, with MHC Class-II shown to be expressed under inflammatory conditions (Franco et al., 1988). Through this, hepatocytes have been shown to be able to activate naïve T cells (Bertolino et al., 2002; Warren et al., 2006).

5.1.2 Adaptive immune responses in HBV-infected liver

Several clinically important pathogens have evolved to target the liver and exploit this tolerogenic environment to establish persistent infection, including HBV and HCV. In opposition to the blood, the liver is selectively enriched for CD8 rather than CD4 T cells. Intrahepatic CD8 T cells are dominated by memory subsets, with significantly fewer naïve T cells infiltrating the liver, compared to the blood.

The liver is also enriched for non-conventional T cells, including mucosal associated invariant T cells (MAIT cells). MAIT cells are identified by their high expression of the C-type lectin, CD161, in association with a semi-invariant TCR restricted by MHC Class-Ib molecule, MR1 (Ussher et al., 2018). These cells represent a transcriptionally distinct lineage, characterised by the expression of RORγT (Billerbeck et al., 2010), and preferentially accumulate in the liver, compared to the blood or gut. MAIT cells are activated via their T cell receptor, which recognises bacterial riboflavin derivatives, or independently via viral infection or TLR-8 agonists. Other non-conventional T cells enriched within the liver include γδ-T cells, which share features of innate and adaptive lymphocytes (Hammerich and Tacke, 2014).

Intrahepatic CD8 T cells constitute the major cell type involved in protecting the liver against pathogens. Recruitment of CD8 T cells to the liver is mediated via the expression of chemokine receptors, including the liver homing chemokine CXCR6 that binds CXCL16 constitutively expressed by liver sinusoidal endothelial cells (Sato et al., 2015). Once within the liver vasculature, HBV-specific T cells adhere to the liver parenchyma through binding to activated platelets via interactions with CD44 on activated CD8 T cells (Guidotti et al., 2015b; Iannacone et al., 2005). In

doing so, their transit through the vasculature is slowed down, allowing CD8 T cells to patrol the sinusoidal vasculature and probe hepatocytes through the extension of cytoplasmic protrusions for the presence of pathogens, elegantly demonstrated by intra-vital imaging (Guidotti et al., 2015b).

Patients controlling acute HBV infection are able to mount an effective virus-specific T cell response; in contrast, HBV-specific responses are markedly depleted and impaired in the presence of chronic HBV-infection. Intrahepatic HBV-specific T cells were shown to have high expression of Bim (Lopes et al., 2008) - a pro-apoptotic factor induced when T cells are primed in the liver as opposed to the lymph node, and that mediates their premature death (Holz et al., 2008). Intrahepatic CD8 T cells can be further depleted through interactions with liver-infiltrating and liver-resident NK cells, which upregulate the death ligand TNF-related apoptosis-inducing ligand (TRAIL), enabling them to kill HBV-specific T cells expressing high levels of the death receptor, TRAIL-R2 (Peppa et al., 2013; Stegmann et al., 2016). Moreover, liver-infiltrating CD4 T cells, in the context of HBV infection, upregulate the expression of stress-ligands, which bind to the NKG2D receptor on NK cells and further promote their activation and cytotoxicity (Huang et al., 2017).

Intrahepatic HBV-specific T cells that persist are profoundly dysfunctional and express high levels of co-inhibitory receptors, in particular PD-1 (Boni et al., 2007; Fisicaro et al., 2010), associated with reduced expression of IFNγ (Schurich et al., 2013). Hepatocytes, KCs and LSECs highly express PD-1 ligands, which are upregulated further in settings of disease (Kassel et al., 2009). Through this interaction, liver-resident cells are able to suppress the function of infiltrating T cells. Additional mechanisms of intrahepatic T cell suppression include the accumulation of specialised inhibitory cells, termed granulocytic myeloid-derived suppressor cells (MDSCs). These are expanded in the livers of patients with HBV infection and produce high concentrations of the enzyme, arginase-1. This enzyme catabolises L-arginine, an essential amino acid required for T cell proliferation, resulting in the local deprivation of essential nutrients and the suppression of antiviral T cell function (Pallett et al., 2015). Despite this, recent work has identified a population of tissue-resident T cells that are specialised to overcome local constraints and preferentially expand in patients with HBV immune control to mediate immunity (Pallett et al., 2017).

5.1.3 Intrahepatic B cells

Comparatively, very little is known regarding intrahepatic B cells. B cells are estimated to constitute approximately 5-10% of intrahepatic lymphocytes in the human liver, contrasting with up to 50% of murine intrahepatic lymphocytes (Racanelli and Rehermann, 2006). However, estimations of B cell frequency within the human liver may be underestimated as a result of relative difficulties in isolating intrahepatic lymphocytes. Work by Steinert et al., identified that isolation methods fail to uniformly isolate the majority of intrahepatic cells, with significant discrepancies demonstrated

between immunofluorescent approaches and flow cytometric quantitation (Steinert et al., 2015). Cell isolation efficiency was markedly affected by tissue type and lymphocyte localisation, suggesting that it may be more difficult to isolate cells that localise deep within liver parenchyma. Thus, challenges associated with the isolation of lymphocytes may account for reduced frequencies of intrahepatic B cells estimated in human livers.

In mice, IgM^+ hepatic B cells are able to efficiently phagocytose fluorescently-labelled *E. coli,* and display bactericidal properties comparable to KCs (Nakashima et al., 2012). Murine hepatic B cells appear also to be potent producers of pro-inflammatory cytokines. Zhang et al., showed that following *in vivo* LPS stimulation, intrahepatic B cells produce significantly more inflammatory cytokines (IL-6, IFNγ and TNFα) compared to splenic B cells, yet displayed reduced secretion of the immunosuppressive cytokine, IL-10 (Zhang et al., 2013). In this system, intrahepatic B cells also promoted the maturation and function of liver myeloid dendritic cells; accordingly, intrahepatic dendritic cells from mice devoid of B cells were less responsive to LPS and showed a decreased ability to produce IL-6, IL-12 and IFNγ. This was in contrast to splenic B cells, which were able to suppress the function of myeloid dendritic cells (Zhang et al., 2013). Combined, these studies from murine models suggest that B cells may have a role in promoting inflammatory responses in the liver.

In contrast, very few studies have explicitly investigated B cells in the human liver due to limitations in access to tissue. B cells have indirectly been shown to have a role in the improvement of primary biliary cirrhosis following B cell depletion via Rituximab (Tajiri et al., 2013). In line with this, an infiltration of B cells and plasma cells, particularly around biliary structures, has been demonstrated in both primary biliary cirrhosis and primary sclerosing cholangitis, purportedly playing a role in the destruction of intrahepatic bile ducts (Fischer et al., 2014; Jin et al., 2012; Zhang et al., 2010).

Intrahepatic B cells may also play an important role in the control of hepatic infections through their capacities to produce antibody, secrete cytokines and present antigen. A recent report identified IgA^+ B cells within the liver that robustly produced antibodies against intestinal antigens (Moro-Sibilot et al., 2016). In chronic viral infections, monoclonal B cells have been shown to infiltrate the liver (Fan et al., 2009), where they can be identified within the portal tracts of patients with HCV (Ward et al., 2007). Due to their localisation and capacity as antigen-presenting cells, intrahepatic B cells are also able to activate liver-infiltrating MAIT cells, which co-localise with B cells within the portal tract. Following exposure to *E. coli*, intrahepatic B cells were demonstrated to activate liver-infiltrating MAIT cells, inducing their degranulation and expression of IFNγ, TNFα, and CD40-L (Jeffery et al., 2016). Thus, in their ability to secrete antibodies and cytokines, alongside their capacity for antigen presentation, B cells are likely important influencers of local inflammatory responses within the liver.

In agreement with murine studies above, accumulating evidence suggests that an influx of B cells to the liver may be associated with increased inflammation and associated damage. $CD20^+$ B cells have previously been identified in the liver of HBV-infected liver, where they were increased in patients displaying a higher degree of liver fibrosis (Mohamadkhani et al., 2012, 2014). Moreover, gene array data comparing HBV-acute liver failure (ALF) to HBV-negative livers showed an upregulation of B cell associated transcripts in ALF. This was associated with an influx of plasma cells to the liver, resulting in overwhelming production of IgM and IgG targeted against HBcAg (Farci et al., 2010). To our knowledge, this example is the only evidence of antigen-specific B cell responses within the liver, thus far.

5.1.4 Evidence for atMBCs in tissue

The tissue localisation of atMBCs, and their role in disease, is undefined at present and warrants investigation. The first description of $FcRL4^+CD21^{low/-}$ B cells in the tonsils implicated a potential role for $CD27^-CD21^{low/-}$ as a tissue-biased B cell population (Ehrhardt et al., 2005). In line with this, FcRL4 expressing cells have been identified in the intraepithelial and sub-epithelial regions of mucosal-associated lymphoid tissue (Falini et al., 2003). It could therefore be postulated that atMBCs represent a tissue-adapted cell type with specialised function in barrier organs.

Phenotypic analysis presented in Chapter Four identified an array of markers specific to atMBCs that might support their identification in the liver. $CD11c^+CXCR3^+$ B cells have previously been shown to localise within the bronchoalveolar space and synovial fluid in patients with rheumatoid arthritis, demonstrating a predisposition of atMBCs towards inflammatory environments (Rakhmanov et al., 2009). CXCR3 has also been demonstrated to direct lymphocyte migration to inflamed liver: CXCR3 ligands are upregulated on hepatic endothelium and facilitate the migration and adhesion of $CXCR3^{hi}$ liver-infiltrating T cells (Curbishley et al., 2005). Similarly, liver-specialised tissue-resident CD8 T cells express comparatively high levels of CXCR3 compared to intrahepatic and peripheral lymphocytes, suggestive of a role for its expression in their homing/retention in the liver (Pallett et al., 2017). Thus, elevated expression of CD11c and CXCR3 on atMBCs and HBsAg-specific B cells may support their homing to inflamed tissue sites.

In addition, we previously showed that atMBCs could be identified by high expression of T-bet. Recent work has begun to identify factors that promote the expression of T-bet in B cells. A combination of TLR-stimulation, in association with Th-1 cytokines (IFNγ and Il-21), was demonstrated to play an important role in the induction of T-bet (Naradikian et al., 2016a; Obeng-Adjei et al., 2017; Sindhava et al., 2017); all key features of HBV-infected liver. Due to the apparent overlap in phenotype between $T\text{-}bet^+$ B cells and atMBCS, it is likely that TLR stimulation, in combination with pro-inflammatory cytokine stimulation, may modulate the transcription of T-bet, leading to the induction of T-bet- and atMBC- associated characteristics. Thus, not only might

atMBCs preferentially migrate to the liver, but the local microenvironment of the liver may induce a dysfunctional phenotype in liver-infiltrating B cells. Therefore, the unique receptor profile of atMBCs, in combination with the complex microenvironment of the HBV-infected liver, may promote an accumulation of HBsAg-specific atMBCs at the site of viral infection.

5.2 Hypothesis and aims:

In this chapter, I aim to examine how the liver microenvironment, in combination with HBV-infection, may influence the frequency and phenotype of liver-infiltrating B cells. Phenotypic analysis presented in the previous chapter indicated that atMBCs and HBsAg-specific B cells in patients with CHB express receptors that would support their homing to inflamed tissues sites, namely CXCR3 and CD11c. We hypothesised that liver-infiltrating B cells may exhibit an atMBC phenotype as a result of their chronic exposure to antigen, in combination with TLR-stimulation and the local cytokine milieu of HBV-infected liver. Finally, using our HBsAg-bait system, we aimed to investigate whether HBsAg-specific B cells are able to persist in HBV-infected liver samples. These data would have important implications in understanding the role of antigen-specific B cells at the site of viral replication and disease pathology.

Using our unique access to liver tissue, we set out to:

1. Examine the frequency and phenotype of atMBCs in paired blood and liver samples, comparing healthy and HBV-infected samples;
2. Assess whether HBsAg-specific B cells are able to persist at the site of viral infection;
3. Dissect the phenotype of HBsAg-specific B cells using parameters presented in Chapter Four.

5.3 Results

5.3.1 B cells with an atMBC phenotype are enriched in HBV-infected and uninfected liver

We postulated that the microenvironment of HBV-infected liver may support an accumulation of exhausted atMBCs at this site. Through unique access to human liver tissue, we were able to assess the effect of both the liver microenvironment and HBV-infection on intrahepatic B cell populations. To do so, we compared lymphocytes extracted from liver biopsy tissue from HBV-infected patients (deemed surplus to diagnostic requirements) to healthy margins of metastatic cancer (referred to as cancer margins). These margins are histologically normal and distal to the tumour, but have the caveat that they are taken from patients with underlying disease and differing treatment regimes. In addition, previous comparison of lymphocytes extracted from these and truly healthy tissue (obtained through biopsy of livers prior to transplantation) has demonstrated subtle alterations in immune composition, likely resulting from distant influences of metastases.

Our analysis also utilised perfusion liquid of cadaveric liver allografts (referred to as perfusates). Analysis of perfusates is arguably biased towards sampling of the lymphocyte populations situated within the liver sinusoids; however, careful characterisation of these samples, and comparison to biopsy tissue, has demonstrated that perfusates are able to reliably sample tissue resident populations, including tissue resident T and NK cells (Pallett et al., 2017; Gill and Pallett., 2018).

To begin, we first analysed the frequency of total B cells ($CD3^{-}CD20^{+}CD19^{+}$) as a proportion of intrahepatic lymphocytes (defined by expression of CD45). B cells were decreased in HBV-infected liver, relative to healthy perfusates or cancer margins (Figure 5.2A). This is likely reflective of a significant influx of infiltrating NK cells and T cells into the HBV-infected liver, decreasing the relative frequency of intrahepatic B cells as a percentage of total intrahepatic lymphocytes. Similar findings were also reported using a separate cohort of liver biopsies (Gill and Pallett., 2018).

We next assessed the proportion of intrahepatic B cells with an atMBC phenotype, to investigate whether atMBCs are preferentially enriched within the B cell pool in the liver environment. Paired *ex vivo* analysis of 27 paired blood and liver samples from patients with CHB or HBV-unexposed patients (healthy margins of metastatic cancer), revealed that the atMBC phenotype observed in the global B cell compartment in the periphery was exaggerated in the liver, compared to the periphery (Figure 5.2B). Comparison of B cells extracted from HBV-infected livers and healthy perfusates showed that atMBCs were further increased in the instance of HBV infection (Figure 5.2C), with healthy margins demonstrating intermediate frequencies of atMBCs. These data pointed to a combined effect of the liver milieu and the virus in the expansion of this subset. Patients with HBV-associated end-stage liver failure (shown in red) had markedly higher levels of atMBCs, suggesting that an accumulation of atMBCs could be associated with worse disease; however, many more patients are required to investigate this concept in full. Interestingly, comparison to a small

subset of HBV-naïve liver biopsies taken from patients with non-viral hepatitis (serum ALT levels ranging from 53-508) did not show as high frequencies as the HBV-infected cohort, suggesting that liver inflammation *per se* was not the sole driver of intrahepatic atMBCs (Figure 5.2C).

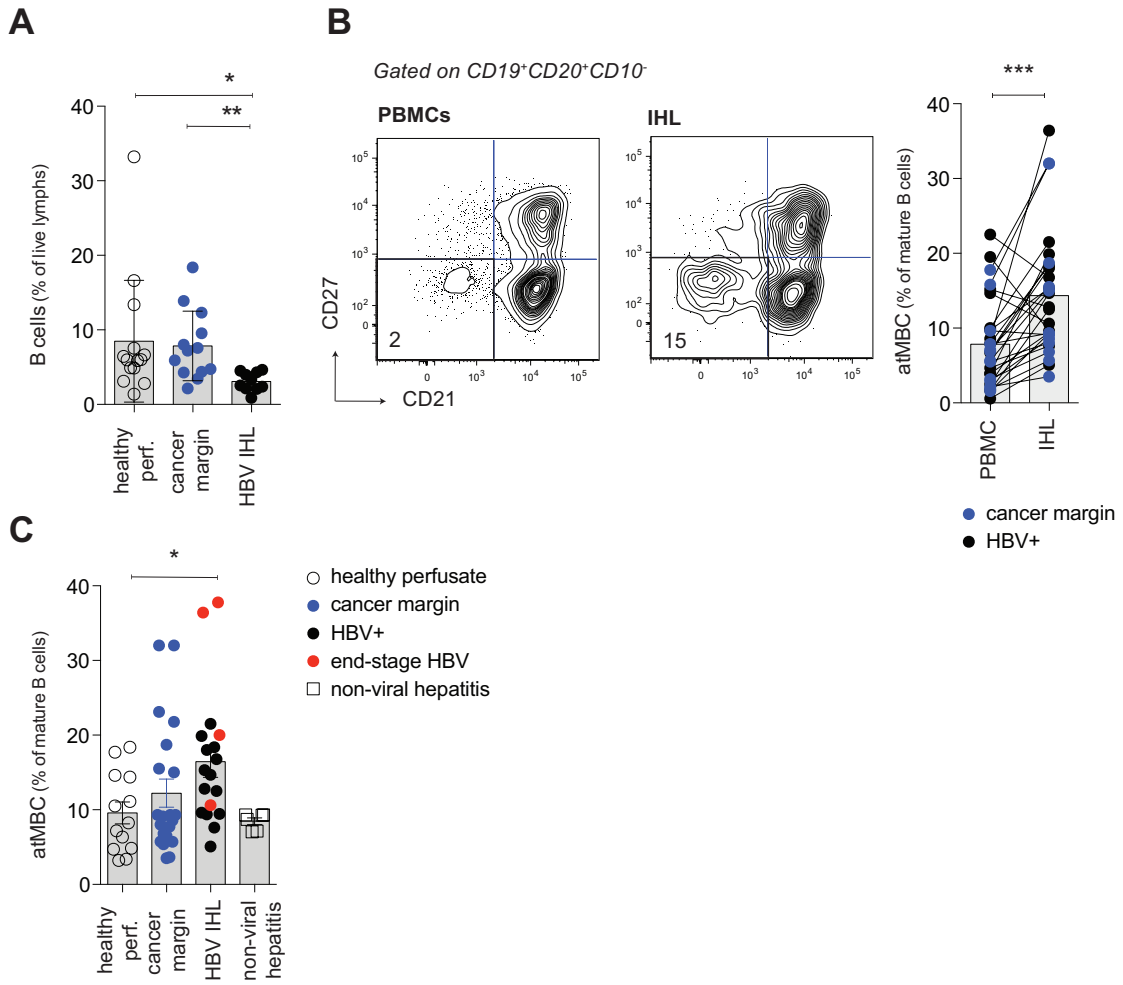

Figure 5-2 atMBCs are increased within the intrahepatic B cell pool compared to the periphery

A. Frequencies of total B cells as a % of live CD45+ lymphocytes. **B.** Representative staining and summary plot: frequencies of atMBC in paired intrahepatic leukocytes (IHL) and blood samples (PBMC) from patients with CHB (black dots; 10 HBV$^+$ liver biopsies; 5 HBV$^+$ liver resections) and uninfected controls (blue dots; 12 margins from HBV- colorectal metastases; CRC; control IHL). **C.** Frequencies of atMBC in control liver samples (22 CRC margins; 12 pre-transplant perfusates; 6 biopsies from livers with non-viral hepatitis) HBV-infected liver (11 HBV$^+$ liver biopsies; 1 perfusate from HBsAg$^+$ liver; 2 perfusates from HBV-resolved livers) including 4 HBV+ explants from HBV-associated end stage failure (shown in red); and 6 biopsies from non-viral hepatitis. Error bars indicate mean ± SEM; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; p-values were determined by determined by Kruskal-Wallis test with a Dunn's post hoc test for pairwise multiple comparisons (a and c); and Wilcoxon signed-rank test (b).

5.3.2 Intrahepatic atMBCs are PD-1^{hi}

The expansion of CD27^{-}CD21$^{low/-}$ B cells in HBV-infected liver led to the hypothesis that exhausted B cells may accrue in the diseased liver as a result of persistent antigenic exposure. Therefore, we next examined the phenotype of intrahepatic atMBCs, to determine whether these cells have comparable attributes to those described in the periphery.

Similar to the circulating atMBCs, intrahepatic atMBCs expressed high levels of the transcription factor T-bet compared to cMBCs, and accordingly accounted for a high proportion of T-bet^{hi} intrahepatic B cells (Figure 5.3A). Intrahepatic atMBCs also expressed higher levels of FcRL5 and PD-1 compared to their counterpart cMBCs (Figure 5.3B), reflecting their dysfunctional phenotype observed in the periphery.

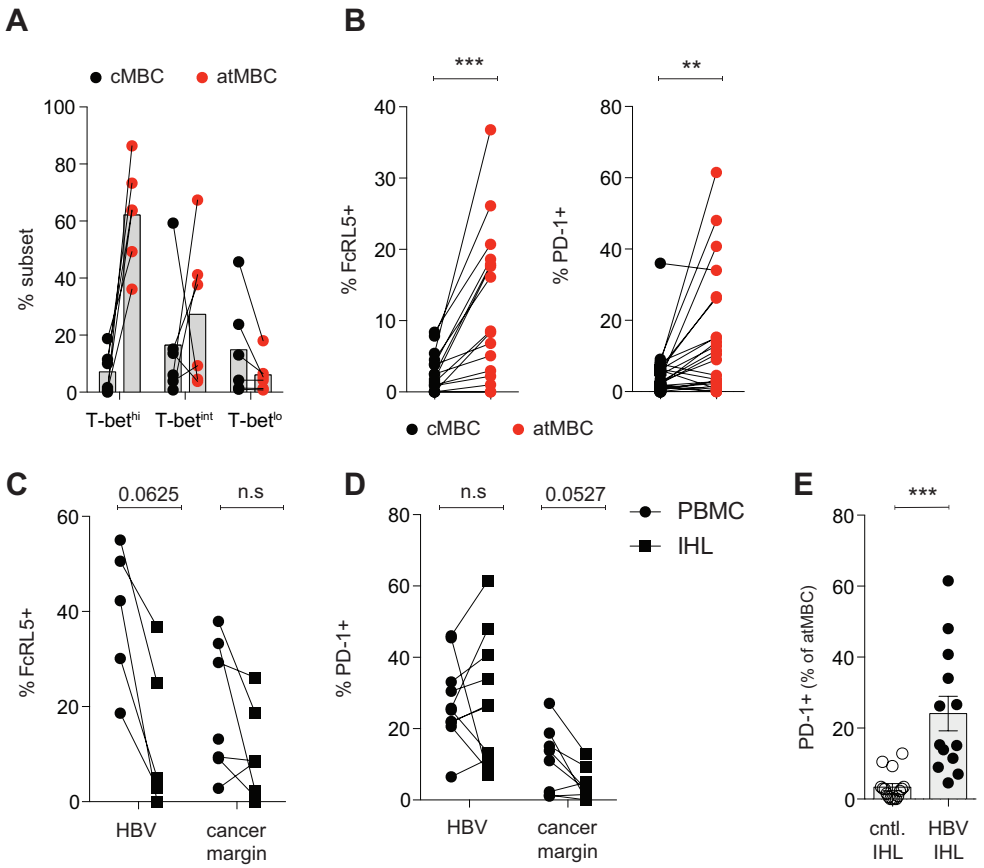

Figure 5-3 Intrahepatic atMBCs phenotypically resemble those in the periphery

A. Percentage of intrahepatic B cells with atMBC or cMBC phenotype within global T-bet^{hi}, T-bet^{int} or T-bet^{lo} populations (n=2 CRC; 3 HBV^{+} tissue; 1 healthy perfusate). **B.** Expression of FcRL5 (n=4 HBV^{+} infected liver; 12 CRC margins) and PD-1 (n=12 HBV^{+} liver; 13 CRC margins) on intrahepatic atMBC and cMBC. **C.** Comparison of FcRL5 expression on atMBCs between the peripheral blood mononuclear cells (PBMC) and intrahepatic lymphocytes (IHL) in HBV-infected tissue (n=4) and cancer margins (n=6). **D.** Comparison of PD-1 expression on atMBCs between PBMC and IHL in HBV-infected tissue (n=9) and cancer margins (n=7). **E.** Comparison of PD-1^{+} atMBC in uninfected liver (%; n=15 CRC margins) and HBV^{+} liver (n=13). Error bars indicate mean ± SEM; **, $P < 0.01$; ***, $P < 0.001$; p-values were determined by determined by Wilcoxon signed-rank test (b, c and d); and Mann-Whitney *U* test for unpaired data (e).

Limited comparison of the levels of FcRL5 on peripheral and intrahepatic atMBCs showed that FcRL5 expression was decreased on intrahepatic B cells, compared to those isolated from the periphery (Figure 5.3C). In contrast, PD-1 levels between intrahepatic and peripheral atMBCs showed that PD-1 expression was comparable within HBV-infected patients, but showed a decrease on intrahepatic B cells in patients with metastatic cancer relative to the periphery (Figure 5.3D). Of note, PD-1 expression was especially high on atMBCs in HBV-infected liver: a maximum of 62% (mean of 28%) of atMBCs expressed PD-1, contrasting to a mean of 3% in uninfected livers (Figure 5.3E). This suggests that the high expression of PD-L1/L2 by liver-resident cell types may contribute to impairing antiviral B cell responses at the site of viral replication. Combined, these data suggested HBV infection, in combination with the unique microenvironment of the liver, may drive an accumulation of atMBCs with similar phenotype features to those identified in the blood.

5.3.3 HBsAg-specific can localise in HBV-infected liver

Since HBsAg is secreted in high quantities by infected hepatocytes, we were next interested to investigate whether HBsAg-specific B cells are able to persist at the site of infection. Expression of CXCR3 and CD11c on HBsAg-specific B cells in the periphery suggested that HBsAg-specific B cells may possess the capacity to home to the liver. To investigate whether HBsAg-specific B cells can infiltrate and persist at the site of viral replication, we stained HBsAg-specific B cells, in a subset of liver samples with sufficient numbers of cells, using the aforementioned HBsAg-bait protocol. As in the periphery, a threshold level (mean plus standard deviation of uninfected controls) was set to determine detectable populations of HBsAg-specific B cells (0.10%). HBsAg-specific B cells were detectable in 12/14 HBV-infected livers sampled, above that seen in HBV-unexposed livers (Figure 5.4A). HBsAg-specific B cells were not at a higher percentage of total B cells in the liver compared to the blood, apart from in one patient who had HBV-associated hepatocellular carcinoma (Figure 5.4B). As seen in the periphery, HBsAg-specific B cells were also enriched for an atMBC phenotype (Figure 5.4C), suggesting that the high levels of circulating HBsAg in the liver may drive an expansion of exhausted B cells within the HBsAg-specific pool. Collectively, these data show that virus-specific B cells can localise at the site of infection, allowing their phenotype and function to be shaped by both the local microenvironment and viral replication.

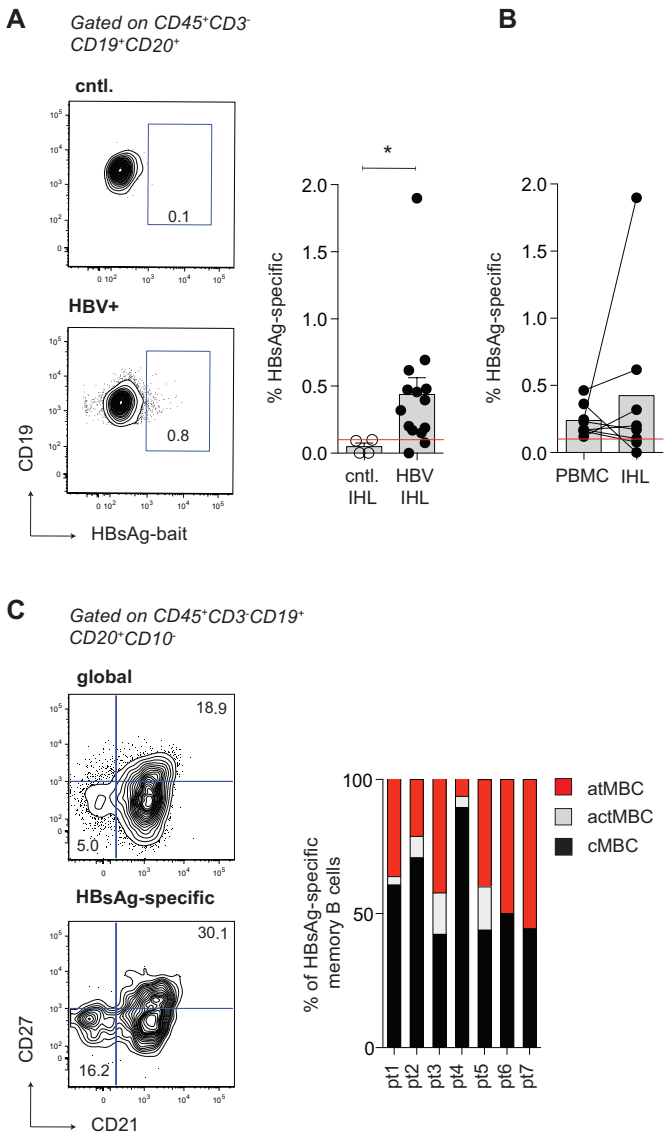

Figure 5-4 HBsAg-specific B cells can persist in HBV-infected liver

A. Representative staining and frequency of HBsAg-specific B cells (% of CD19+CD20+) in HBV+ liver samples (n=14) compared to uninfected controls (n=4 CRC margins). **B.** Frequency of HBsAg-specific B cells in paired PBMC and liver samples. **C.** Representative staining and frequencies of HBsAg-specific memory B cell subsets in 7 individual HBV+ liver samples (pt1-pt7). Error bars indicate mean ± SEM; *, P < 0.05; **, P < 0.01; ***, P < 0.001; ****, P < 0.0001; p-values were determined by Mann-Whitney *U* test for unpaired data (a); and Wilcoxon signed-rank test (b).

5.3.4 Activated HBsAg-specific B cells can be identified in liver-draining lymph node

Our data thus far has identified HBsAg-specific B cells in the blood and liver of patients with CHB. However, by limiting our analyses to these samples, we are potentially underestimating the true frequency of HBsAg-specific B cells that may additionally be concentrated (or compartmentalised) within lymphoid tissue. Through our close collaboration with the Royal Free surgical and histopathological teams, we were able to obtain matched samples of blood, liver and a liver-draining lymph node from a patient with end-stage HBV infection, facilitating limited analysis of HBsAg-specific B cells in liver-associated lymphoid tissue.

HBsAg-specific B cells (HBsAg-bait^{+}CD20^{+}CD19^{+}) were detected *ex vivo* at similar frequencies in the blood, liver and lymph node (Figure 5.5A). When phenotyped in more detail, preliminary analysis in the lymph node revealed a population of HBsAg-specific Ki67^{+}CD38^{hi} B cells, indicative of highly activated and cycling B cells that were present in the lymph nodes and absent in the liver and PBMCs (Figure 5.5B). These cells may putatively represent germinal centre B cells; however, additional makers - including expression of the master regulator of germinal centre B cells, Bcl-6 (Basso and Dalla-Favera, 2010) - are required to confirm this phenotype. Notably, Ki67^{+}CD38^{hi} B cells were identifiable in the liver, however were not specific for HBsAg.

We next analysed whether any HBsAg-specific plasma cells/blasts (HBsAg-bait^{+}CD20^{-}CD19^{+}) were present in each sample. HBsAg-specific plasma cells/blasts were noticeably present in the liver and lymph node, but absent in PBMCs. Strikingly, up to 97.7% of plasma cells/blasts in the lymph node were specific for HBsAg, compared to 37.7% in the liver (Figure 5.6A). These cells expressed high levels of plasmablast markers, including Blimp-1, CD80, CD86 and Ki67, and were negative for expression of CD20 (Figure 5.6B) (Fink, 2012). Combined, these data point to the presence of HBsAg-specific germinal centres in liver-adjacent lymph nodes, and to a lesser extent in liver tissue itself, resulting in the formation of HBsAg-specific plasmablasts. However, without the addition of the plasma cell marker, CD138, we are unable to conclude whether these cells represent longer-lived plasma cells; further analysis would be required to dissect whether any of these cells have the capacity to form long-lived plasma cells, or whether the cells identified represent short-lived plasmablasts. However, this analysis sets a precedent for analysing HBsAg-specific plasma cells/blasts in future HBV-infected liver samples.

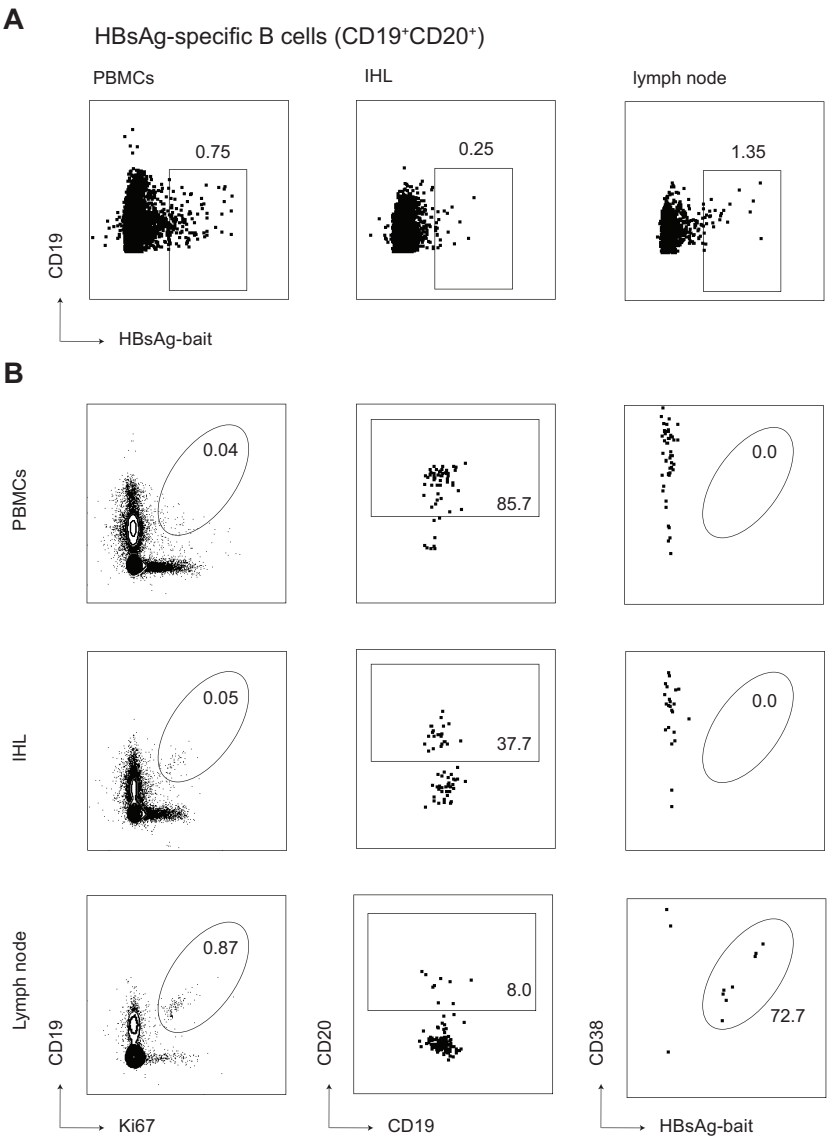

Figure 5-5 Cycling HBsAg-specific B cells detected in the draining lymph node in HBV-infected livers

A. Frequency of HBsAg-specific B cells ($CD20^+CD19^+$) in the blood, liver and draining lymph node of a HBV-infected liver. **B.** Sequential flow cytometric plots of HBsAg-specific B cells with features of germinal centre B cells in the draining lymph node (absent in the blood and liver). Cycling B cells were identified by expression of Ki67 and CD19. Cycling plasmablasts were excluded through expression of CD20. Putative HBsAg-specific germinal centre B cells were identified by their expression of CD38 and binding to HBsAg-bait. Numbers in plots represent the gated population as a % of the parent population.

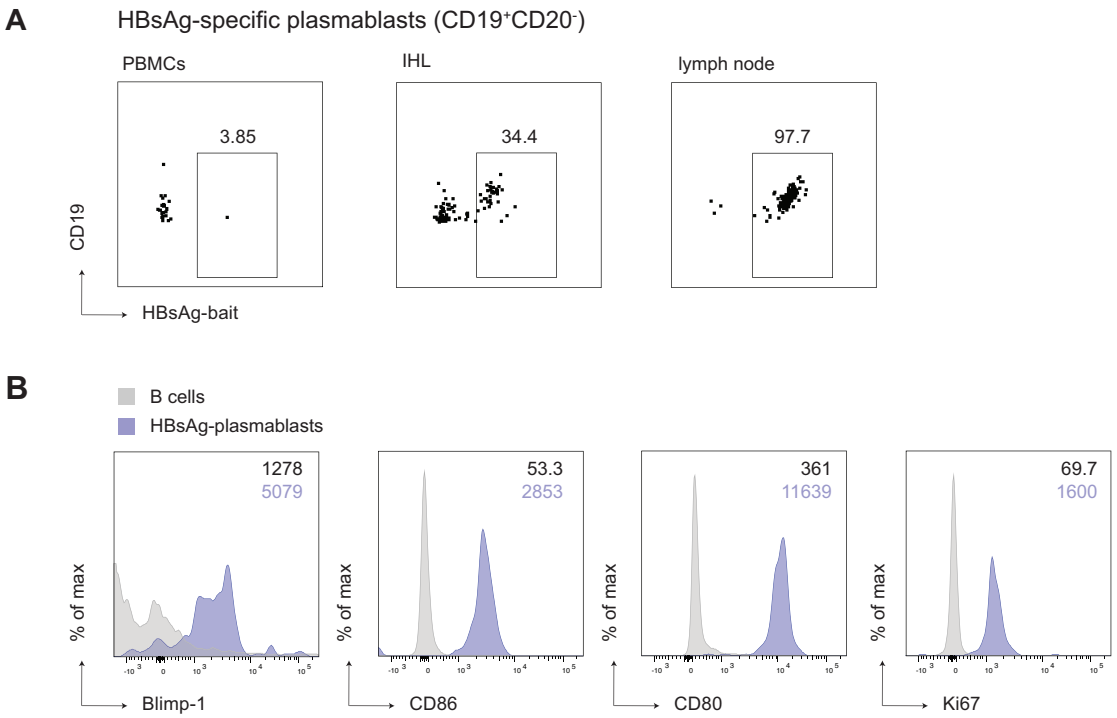

Figure 5-6 Evidence of HBsAg-specific plasmablasts in the draining lymph node in HBV-infected liver

A. Frequency of HBsAg-specific plasmablasts ($CD20^{-}CD19^{+}$) in the blood, liver and draining lymph node of a HBV-infected liver. **B.** Flow cytometric plots demonstrating the expression of plasmablast markers on HBsAg-specific plasmablasts in the lymph node (identified as HBsAg-$bait^{+}CD20^{-}CD19^{+}$; purple) compared to B cells ($CD20^{+}CD19^{+}$; grey). Numbers in plots represent the gated population as a % of the parent population (a) or corresponding MFI (b).

5.4 Discussion

In this chapter, we describe an increased proportion of intrahepatic B cells with an atMBC phenotype, relative to paired blood samples. These cells phenotypically resemble those seen in the periphery, but in particular, demonstrate elevated expression of PD-1 and T-bet. Through comparison of HBV-infected livers to uninfected livers, we demonstrate that HBsAg-specific B cells are able to persist at the site of infection, yet acquire an atMBC phenotype similar to the periphery. In particular, we show preliminary data indicating that HBsAg-specific B cells can also be identified in liver-adjacent lymph nodes, where they represent antigen-experienced populations akin to germinal centre B cells and plasmablasts.

In contrast to HBV-specific T cells, that are enriched within the intrahepatic compared to peripheral T cell compartment (Fisicaro et al., 2010; Maini et al., 2000), HBsAg-specific B cells were not increased in the liver relative to the blood in terms of their proportion within the total B cell compartment. However, it is difficult to interpret this frequency in relation to their absolute number, due to overarching alterations in the composition of the lymphocyte compartment in the liver compared to the blood. In addition, the frequency of B cells may be underestimated depending on their localisation within the liver, as demonstrated by variations in the proportion of cells isolated compared to those quantified in tissue sections (Steinert et al., 2015). Moreover, while their relative abundance is important, there may also be interesting differences in the fine specificities of the antibodies and isotypes produced by intrahepatic B cells, compared to their peripheral counterparts. Hence, it is important to study antigen-specific B cells in both the liver and periphery in more detail.

To our knowledge, this is the first demonstration that human pathogen-specific memory B cells can localise within an infected organ. These data were supported by the finding that HBsAg-specific cells resembling plasmablasts were identifiable in the liver and adjacent lymph node in a patient with HBV-associated end-stage liver failure. The identification of intrahepatic HBsAg-specific B cells has important implications for their roles at the site of viral replication and disease pathology. Firstly, it implies that it may be possible to boost intrahepatic plasma cells capable of producing anti-HBs antibodies with neutralising or other antiviral capacity. Local production of anti-HBs antibodies would facilitate their binding to intrahepatic virions to block infection of new hepatocytes, or to HBsAg on the hepatocyte membrane to promote elimination through antibody-dependent cell-mediated cytotoxicity (ADCC). Immunohistological staining of HBV-infected liver biopsies has demonstrated both cytoplasmic and membranous HBsAg staining, depending on the target epitope of the anti-HBs antibody used, revealing the potential for ADCC-mediated depletion of infected hepatocytes (Eren et al., 1998; Ray et al., 1976). Expression of HBsAg on the plasma membrane is postulated to vary according to disease state, with membranous localisation closely related to active viral replication (Chu and Liaw, 1987). These reports lend strength to the idea that

local production of anti-HBs may facilitate eradication of HBV-infected hepatocytes through ADCC. Moreover, work using a hepatoma cell line has suggested hepatocytes may also be able to internalise anti-HBs antibodies through FcR receptors that could then directly exert intracellular antiviral effects (Schilling et al., 2003).

Analysis of liver-adjacent lymphoid tissue showed that HBsAg-specific plasmablasts, and to a lesser extent, germinal centre B cells, may develop and persist in liver-adjacent lymphoid structures, in line with observations of enlarged hepatic lymph nodes in patients with CHB (Choi et al., 2001; Kuo et al., 2006). This observation is perhaps counter-intuitive, since we have previously hypothesised that patients with CHB have a diminished ability to produce anti-HBs. However, interpretation of this data has some important caveats. Firstly, the lack of additional samples limits the context with which we have to interpret the data; for instance, we are not able to determine the relative abundance of these cells compared to other samples and disease states. In addition, simple phenotyping of these cells is too reductionist. The limited assessment of phenotypic markers cannot conclude whether these cells represent short-lived plasmablasts or longer-lived, high-affinity plasma cells. Finally, we were not able to assess the quality of antibody produced by these cells. Thus, future studies should aim to clone the antibody produced by these cells and assess its neutralising capacity. Overall, these cells may represent a functional population of anti-HBs-producing cells that could be harnessed to promote virus neutralisation at the site of replication.

The detection of HBsAg-specific plasmacytes within the liver tissue itself may also have important inferences for the formation of HBsAg-specific humoral immunity at the site of infection. It has long been described that ectopic lymphoid structures resembling germinal centres can arise in non-lymphoid tissues and assist in the activation, proliferation and differentiation of B cell responses. One such description is the formation of bronchus-associated lymphoid tissue (BALT), identified in infectious settings of the airways and characterised by a defined B cell follicle containing blasting, $Ki67^{hi}$ B cells (Randall, 2010). BALT is postulated to assist in the promoting of local antiviral responses: for example, influenza infection rapidly induces the formation of BALT, capable of priming antigen-specific T and B cell responses independently of primary lymphoid organs (Moyron-Quiroz et al., 2004). Emerging evidence suggests that persistence of germinal centre responses may have a role in boosting protection from highly mutating viruses, such as influenza. Lung germinal centres support B cell proliferation and the generation of neutralising antibodies, and facilitate fine-tuning of B cell responses by antigen and T_{FH} cells (Adachi et al., 2015). The identification of these structures in the lung has overturned the dogma that antigen-specific responses are solely initiated in lymphoid tissue and suggests that tissue localisation of B cells may provide an important mechanism for antiviral control.

Thus, the identification of cycling plasmacytes within liver tissue could arguably elude to the formation of ectopic germinal centres in the form of intrahepatic lymphoid follicles (ILFs). ILFs have been described in the livers of patients with autoimmune hepatitis, primary biliary cirrhosis and CHB, yet are most highly reported in HCV infection (Lefkowitch et al., 1993). Here, ILFs with features of ectopic germinal centres (including Bcl-6 and Ki67) are postulated to facilitate expansion of both HCV-specific and autoreactive clones (Murakami et al., 1999; Tucci et al., 2014). Thus, the existence of these structures may have unique importance to HCV, due to the occurrence of B cell-associated lymphoproliferative disorders, including mixed cryoglobulinemia and B cell non-hodgkin lymphomas that arise in a subgroup of HCV-infected patients.

In contrast, a role for ILFs in CHB has largely been ignored. In comparison to HCV, patients infected with HBV show little signs of autoimmunity. It also seems unlikely that HBsAg-specific B cells undergo clonal expansion in the liver as ILFs in CHB have been shown to lack a well-formed germinal centre (Murakami et al., 1999), in line with the absence of HBsAg-specific germinal centre B cells in our sample. Due to their apparent role in amplifying tissue-based responses, a lack of intrahepatic germinal centre formation in the livers of patients with CHB may offer a mechanism by which local humoral immunity is impaired. A recent paper testing the immunotherapeutic effects of TLR-7 agonists in HBV-infected chimpanzees demonstrated transient induction of B and T cell aggregates in portal regions following treatment, coinciding with prolonged suppression of serum viral DNA and antigens. Preliminary investigation suggested that these structures did not contain germinal centre reactions; however, the association between the formation of these structures and the antiviral response to treatment, suggested that they may play a role in promoting an effective response against HBV (Li et al., 2018). Further in-depth analysis is required to investigate whether memory B cells can become activated and differentiate within the liver, or traffic to the liver post activation, and how this affects the development and maintenance of local humoral responses.

These data demonstrated a significant expansion of global and antigen-specific atMBCs as a proportion of mature B cells in HBV-infected liver compared to paired blood samples, suggesting that liver-infiltrating B cells are skewed towards an atMBC phenotype at the site of disease pathology. This accumulation of atMBCs may have important consequences for immune pathology. As discussed in detail in previous chapters, a subset of atMBCs express high levels of IgM. This finding raises the possibility that liver-infiltrating B cells, with a naïve-like phenotype, contribute to the immunopathogenesis of CHB. In particular, it will be important to investigate whether the IgM-expressing fraction of atMBC in CHB can synthesise antibodies with pathogenic potential, as previously ascribed to intrahepatic IgM$^+$anti-HBc antibodies in HBV-associated acute liver failure (Farci et al., 2010).

On the other hand, this expansion of atMBCs within the intrahepatic B cell pool, may represent adaptations of liver-infiltrating B cells, in line with the tolerogenic environment of the liver. While there were significantly more atMBCs in HBV^{+} liver, CD27^{-}CD21$^{lo/-}$ B cells also represented a substantial component of the global B cell pool in healthy perfusate samples and margins taken from cancer resections. The liver is exposed to significant amounts of harmless and pathogenic antigen due to its role in the detoxification of products in the portal vein blood, draining directly from the gastrointestinal tract. It could therefore be proposed that intrahepatic B cells of global antigen-specificity acquire this phenotype as a result of excessive antigen exposure in the liver, possibly as a tolerogenic mechanism contributing to the preservation of organ integrity. This is particularly relevant in the context of cytokine secretion, where a reduced capacity to secrete IL-6 and TNFα (demonstrated in Chapter Four) may be helpful in containing a pro-inflammatory response and limiting excessive tissue damage.

In line with this, preliminary data from patients with end-stage liver failure showed high levels of atMBCs cells within the intrahepatic B cell pool. These patients are well-described as having high levels of bacterial infection and endotoxemia in the liver due to worsening of intestinal defence mechanisms (Thalheimer et al., 2005). This is likely to increase both the inflammatory state of the liver and the antigenic burden, and thus may explain the enrichment of atMBCs seen. However, patients with non-viral hepatitis, but high levels of ALT, demonstrated levels of atMBCs similar to that of healthy samples, suggesting that inflammation alone has a limited effect on the expansion of atMBCs in the human liver. Therefore, it could be postulated that increased exposure to antigen is a primary driver of atMBCs in the liver. Although atMBC could be identified in non-HBV infected livers, they were more frequent in those infected with HBV and expressed more PD-1 in the latter group. These findings, from *ex vivo* examination of B cells freshly isolated from a large number of human liver samples, therefore support a combined effect of the tolerogenic liver milieu and HBV viral antigen in driving the atypical and PD-1^{hi} phenotype.

Overall, these data point to a previously unappreciated potential for a role of antigen-specific B cells in HBV-immune control at the site of viral replication. Intrahepatic HBsAg-specific memory B cells were enriched for an atMBC phenotype, similar to findings of peripheral HBsAg-specific B cells, and in line with high levels of antigen and inflammation in HBV-infected liver. Thus, immunotherapeutic approaches to boost B cell responses in CHB should consider direct targeting of therapies to intrahepatic B cell populations to maximise efficacy and harness B cell effector functions at the site of viral replication. Finally, the identification of HBsAg-specific B cells in liver-adjacent lymph node suggests that there may be alternative populations of HBsAg-specific B cells whose functional contribution to intrahepatic immune responses should now be considered in detail.

Chapter 6 Discussion and future directions

6.1 Thesis conclusions

Ongoing work in the field of HBV aims to understand the multi-faceted failure of the immune response in the control of HBV and develop new immunotherapeutic strategies to induce functional cure in patients with CHB. The rationale for this approach is supported by the observation that most adults infected with HBV resolve the infection naturally, and are able to maintain the virus under lifelong immune control. It has long been recognised that patients with CHB fail to produce sufficient levels of protective anti-HBs antibodies to overcome the significant quantities of circulating HBsAg released by infected hepatocytes. However, investigation of B cell and humoral responses in CHB has been significantly neglected. In this study, I have for the first time analysed the phenotype and function of HBsAg-specific B cells in both the blood and the liver of patients with CHB. I demonstrate an accumulation of B cells with defective antiviral function within the global and antigen-specific population, in both the circulation and liver of patients with CHB. I postulate that this phenomenon may contribute to impaired antiviral responses observed in CHB, and provide new targets for immunotherapeutic therapies aimed at achieving functional cure in HBV.

One of the key strengths of this study is the utilisation of well-characterised clinical samples, permitting careful description of antigen-specific responses, both in chronic infection and longitudinally throughout acute-resolving HBV. In studying these samples, I have been able to compare the frequency and phenotype of HBsAg-specific B cells according to the disease status of the individual, revealing a number of key findings. Firstly, I showed that HBsAg-specific B cells persist in the circulation of patients with chronic infections at similar frequencies to those with resolving infection and approximating levels reached by protective HBV-vaccination. However, HBsAg-specific B cells demonstrated defective production of anti-HBs antibodies, relative to HBV-vaccinated healthy controls. This allowed me to hypothesise that there is not a defect in the numerical differentiation or formation of HBsAg-specific B cells, but instead that these cells are dysfunctional and incapable of producing sufficient levels of protective anti-HBs antibody.

Secondly, comparison of HBsAg-specific B cells between vaccinated healthy controls and patients with CHB revealed a number of ways in which HBsAg-specific B cells may be impacted by chronic HBV infection. *Ex vivo* staining showed that HBsAg-specific B cells from patients with CHB had elevated expression of inhibitory receptors, PD-1 and FcRL5, alongside a number of phenotypic markers reflective of an accumulation of "dysfunctional" atMBCs. Patients with CHB also had increased frequencies of global atMBCs, relative to vaccinated healthy controls. Further characterisation of these cells suggested that these cells have impaired antiviral function following

triggering of the BCR and CD40 co-stimulation; thus, their accumulation within the HBsAg-specific pool may contribute to decreased antiviral activity against circulating antigen and sub-viral particles. This expansion of supposedly dysfunctional atMBCs within the global as well as antigen-specific B cell population is interesting and mirrors that seen within CD8 T cells, whereby global T cell dysfunction is thought to arise due to chronic inflammatory events and exposure to the inflamed microenvironment of HBV-infected liver (Das et al., 2008; Park et al., 2016; Walker et al., 2013). Preliminary investigation suggested that B cell function could be partially restored through PD-1 blockade in combination with CD40-L stimulation.

Cross-sectional analysis did not reveal a clear correlation of either the frequency of HBsAg-specific B cells or their phenotype to clinical parameters of disease. However, these provide only a snapshot into the impact of disease on B cells. In comparison, careful characterisation of HBsAg-specific B cells throughout acute- and chronic-resolving infection facilitated consideration of the frequency and phenotype of HBsAg-specific B cells throughout the time course of infection. This analysis revealed a tendency for HBsAg-specific B cells and atMBCs (both global and antigen-specific) to decrease towards the resolution of disease. Although I was unable to attribute these variations to a particular component of HBV-infection, I suggest that an expansion of HBsAg-specific atMBCs can be associated with active infection. Moreover, patients with active disease (viral loads >2000IU/ml) showed higher expression of PD-1, suggestive of a role for high levels of HBV DNA in impairing B cell responses.

Finally, access to precious human HBV-infected and HBV-unexposed liver samples has allowed me to characterise antigen-specific B cells at the site of infection; one of the few examples where this has been possible in human non-lymphoid tissue. In particular, I provide evidence that HBsAg-specific B cells are able to persist in the liver; however, their function may be regulated by their anatomical location. Intrahepatic HBsAg-specific B cells showed a similar accumulation of atMBCs to those identified in the periphery, yet HBsAg-specific cells resembling germinal centre B cells and plasmablasts could be identified in the liver-draining lymph node of a patient with end-stage HBV. Crucially, the identification of antigen-specific B cells at the site of viral replication raises prospects for immunotherapeutic targeting of key antiviral effector cells at the site of pathology.

6.2 Unanswered questions and future directions

The work presented within this thesis raises many interesting avenues for future research into the impact of HBV infection on antigen-specific B cell responses and their role in long-term viral control. Although not exhaustive, the following describes some of the key unanswered questions raised by this study, and discusses potential directions for future work investigating B cell responses in CHB.

6.2.1 What is the memory potential of $CD27^{-}CD21^{low/-}$ memory B cells?

$CD27^{-}CD21^{low/-}$ B cells described within this study have been referred to throughout this thesis as atMBCs, due to their similarities in phenotype and function to those reported in the litreature, and in particular, their well-documented expansion in the context of chronic infections. However, this study has not explicitly characterised the memory potential of these cells. Immunological memory is defined as the ability of lymphocytes to respond rapidly upon secondary exposure to their cognate antigen to produce high-affinity antibodies. Many methodologies can be applied to distinguish B cells with memory potential, including: rates of somatic hyper-mutation and cell division (as a proxy of clonal expansion within the germinal centre); isotype class-switching; and increased expression of costimulatory molecules, CD80, CD86 and CD27.

My data also revealed that a significant proportion of atMBCs were $IgM^{+}CD27^{-}CD21^{low/-}$. By conventional definitions, these cells arguably do not represent memory cells and instead may be a naïve-like cell type, as discussed in Chapter Four. Relying solely on such definitions, however, may ignore some important cell types. Recent studies have shown that antigen-specific IgM^{+} memory B cells represent a key effector cell upon secondary exposure to *Plasmodium*. As a long-lived cell type, these cells dominated early secondary responses, and were able to out-compete IgG^{+} memory B cells to produce rapid and plastic humoral responses (Krishnamurty et al., 2016). However, IgM^{+} memory B cells are typically defined by expression of the classical memory marker, CD27, absent on IgM^{+} atMBCs (Seifert and Küppers, 2016). It will now be key to examine the level of somatic hyper-mutation occurring within $IgM^{+}CD27^{-}CD21^{low/-}$ B cells, and determine their memory potential through their ability and rapidity in differentiating into antibody-secreting cells.

6.2.2 Dynamics of atMBCs and/or HBsAg-specific B cells during treatment for HBV

Data presented within this thesis suggest that atMBCs decrease in frequency following resolution of disease. To further support this finding, it would be interesting to examine how HBsAg-specific B cell responses evolve throughout treatment. Studies in HIV infection have shown that the expansion of atMCBs is corrected after prolonged anti-retroviral therapy and subsequent reduction in viraemia (Amu et al., 2014; Muema et al., 2015). Cross-sectional analysis pre- and post-treatment

showed that the proportion of cells with an atMBC phenotype decreased more significantly upon early initiation of ART, and coincided with improved responses to HIV- and non-HIV- antigens (Moir et al., 2010). This effect was postulated to be the result of reduced B cell hyper-activation as a result of lower antigen loads, resulting in lower rates of B cell turnover and apoptosis. Ongoing studies within our group are now assessing the kinetics of B cell subsets in patients undergoing nucleoside withdrawal therapy, in some cases followed by short term pegylated-IFN treatment. Such strategies aim to reactivate immune control and promote HBsAg seroconversion, whilst removing dependency on life-long nucleoside treatment.

However, recent studies in LCMV infection have suggested that type I IFN may have adverse effects on the survival and function of antigen-specific B cells. Early onset type I IFN induced inflammation was postulated to bias antiviral B cell differentiation of naïve B cells into short-lived plasmablasts, mediated by bystander myeloid cells, dendritic cells and T cells (Fallet et al., 2016). Similarly, type I IFN suppressed antibody secretion and impaired the survival of antigen-specific B cells via the recruitment of inflammatory monocytes (Sammicheli et al., 2016), and promoted CD8 T cell deletion of antiviral B cells (Moseman et al., 2016). In all three reports, blockade of IFN signalling significantly boosted antigen-specific B cell survival, resulting in robust production of neutralising antibodies. Therefore, it will be important to monitor the effects of interferon treatment on HBsAg-specific B cell responses.

6.2.3 Additional factors contributing to B cell dysfunction in CHB

It is clear that substandard antibody production by HBsAg-specific B cells in CHB cannot solely be attributed to the atMBC fraction expressing inhibitory receptors. There are likely additional, more generalised B cell defects exerted by the presence of the virus and high levels of circulating antigen. Emerging evidence has identified new molecular targets of suppression arising in B cells during viral infection. One such mechanism, shown to regulate BCR signalling and antibody secretion in B cells, is the upregulation of the inositol phosphatase, PTEN (phosphatase and tensin homolog). PTEN is a tumour suppressor protein and has both lipid and protein phosphatase abilities. Through its negative regulation of the PI3-kinase cascade, PTEN has diverse effects on multiple aspects of cellular homeostasis, including cell proliferation and survival, cell migration and self-renewal of stem cells (Milella et al., 2015). As a result, it has long been known that alterations in levels and activity of PTEN result in an increased susceptibility to various forms of cancer, and often underpin rapid tumour progression (Alimonti et al., 2010; Lee et al., 2018). In HBV infection, HBx protein has been proposed to drive expression of PTEN in an HBV-expressing HepG2 cell line via the upregulation of miRNA-21, resulting in increased hepaotcyte proliferation (Damania et al., 2014). However, subsequent studies have since suggested that this effect was independent of PTEN, instead showing a role for PTEN in promoting HBV replication (Wu et al., 2018).

Following on from this, PTEN expression has been demonstrated to regulate antiviral responses in acute viral infection, reflective of defective responses demonstrated by atMBCs in this study. Infection with gammaherpesvirus-68 upregulated expression of PTEN in murine B cells, which inhibited PI3-kinase signalling and resulted in impaired BCR signalling and CXCR4-mediated migration. Importantly, overexpression of PTEN significantly suppressed antibody production by B cells, demonstrating a previously unidentified pathway of virus-induced immune suppression of humoral responses (Getahun et al., 2017). However, how the virus was able induce PTEN expression in B cells not containing the virus is not yet known.

Thus, in addition to the expansion of dysfunctional atMBCs driven by persistent exposure to high levels of circulating antigen, HBV may also impart additional defects on the functioning of global B cell subsets. A back-to-back study that compared the transcriptome of memory B cell subsets in patients with CHB showed differential expression of genes in all subsets compared to healthy controls, including genes involved in the regulation of NFκB signalling and in the maintenance of T_{FH} - B cell interactions (Salimzadeh et al., 2018). Whilst these were most pronounced within atMBCs, differences were also seen in cMBCs, activated memory B cells and naïve B cells. These data showed that HBV infection does not only alter the composition of B cells within patients with CHB, but can also impart significant differences in gene expression. A recent study has shed light on mechanisms by which HBV viral particles can manipulate B cell responses, whereby HBsAg was shown suppress phosphorylation of the transcription factor CREB and restrict TLR-9 promoter activity. As a result, TLR-9 expression and downstream function was suppressed in B cells from patients with CHB, including proliferation and cytokine production (Tout et al., 2018).

6.2.4 Impact of chronic HBV infection on T_{FH} – B cell interactions

Several lines of evidence within this study lead us to postulate that T follicular (T_{FH}) – B cell interactions may be impaired in CHB, which may have direct effects on the ability of HBsAg-specific B cells to secrete protective antibody. These include:

1. Decreased expression of CD40 on atMBCs, which may impair B cell co-stimulation via CD40-ligand expression on CD4 T cells
2. Decreased expression of CXCR5 on atMBCs, possibly resulting in impaired migration of atMBCs to B cell follicles in lymphoid tissue
3. Decreased levels of class-switching, indicative of impaired T_{FH} help and induction of class-switch recombination/germinal centre responses

The efficiency of T_{FH} – B cell interactions in viral infections is key to the generation of antibody responses. CD4 T cell depletion studies have shown that the induction of a strong antibody response is dependent on an intact CD4 compartment (Vieira and Rajewsky, 1990). Through the

expression of ICOS and CD40-ligand, and production of helper cytokine IL-21, T_{FH} cells support the expansion and differentiation of B cells into long-lived plasma cells or memory cells (Fahey et al., 2011; Harker et al., 2011; Lindqvist et al., 2012). An absence T_{FH} at the onset of LCMV infection curtails the humoral immune response, preventing clearance of the virus (Fahey et al., 2011). However, T_{FH} cells are also important in late stages of persisting viral infection: ablation of $CXCR5^+$ CD4 T cells after the initial establishment of immunoglobulin responses, showed that T_{FH} are critical to the ongoing production of neutralising antibody against the infecting virus and newly emerging variants (Greczmiel et al., 2017). These findings suggest that T_{FH} are central to the evolution of B cell clonality throughout the course of persistent infection.

Accumulating data purport to the dysregulation of T_{FH} responses resulting in aberrant protective B cell responses in chronic infection. Similar to CD8 T cells, HBV-specific CD4 T cells are detected at very low frequency throughout different stages of HBV infection (Penna et al., 1996). HBV-specific T_{FH} have been shown to be increased in CHB compared to healthy controls (Hu et al., 2014), yet some reports exhibit functional exhaustion associated with high expression of PD-1 (Raziorrouh et al., 2014). Dysfunction of CD4 T cells has been partially attributed to an expansion of IL-10-producing B cells in hepatocellular carcinoma (Xue et al., 2016). Along the same line, IL-10-producing B regulatory cells have also been shown to suppress inflammatory responses by promoting the differentiation of T regulatory cells (Tregs) (Flores-Borja et al., 2013). An early study addressing the inability of B cells from HBsAg carriers to produce anti-HBs, demonstrated that co-culture of B and T cells from vaccinated healthy controls with T cells from HBsAg carriers suppressed anti-HBs production (Dusheiko et al., 1983), implicating a role for regulatory T cells in impairing T_{FH}-help and anti-HBs production in CHB. These data are consistent with a recent report of Treg-mediated suppression of HBsAg-specific T_{FH} in HBV, resulting in impaired production of anti-HBs antibodies in a murine model of HBV. *In vivo* blockade of CTLA-4 restored the ability to clear HBV infection in mice, and significantly increased IL-21 production by T_{FH} in patients with CHB, proposing a potential target for the rescue of anti-HBs responses by HBsAg-specific B cells (Wang et al., 2018b). $CXCR5^+CD4^+$ T_{FH} in HBV had a significant expansion of $FoxP3^+CD25^+$ Treg-like T_{FH} cells. These were distinct from $CD25^-$ T_{FH} and secreted lower IFNγ but increased levels of TGF-β. Crucially, $CD25^+$ T_{FH} were less capable of inducing plasmablast differentiation in B cells and instead promoted the differentiation of regulatory B cells (Wang et al., 2018a).

Thus, future studies should analyse HBsAg-specific B cells, Tregs and T_{FH} in parallel to better define the influence of CD4 T cell help on B cell functionality in patients with CHB. During infection, B cells and T_{FH} reside predominantly within the B cell follicles in secondary lymphoid tissues. This localisation is mediated via expression of CXCR5, responding to high concentrations of CXCL13 within the B cell zone. Therefore, investigation of T_{FH} – B cell interactions would be most informative within secondary lymphoid organs or in liver tissue, which may have the potential

to serve as a secondary lymphoid site. Due to the relative difficulty in obtaining these samples from HBV-infected individuals, this would be most likely achieved through immuohistochemical analyses in paraffin-embedded slides. This would facilitate assessment of relative numbers of B and T_{FH} cells, co-localisation of cells and formation of germinal centres, and phenotypic analysis of T_{FH} subsets. Access to fresh tissue, where possible, would permit functional analyses, for example T_{FH} production of IL-21 – the key cytokine involved in driving B cell proliferation and germinal centre responses (Linterman et al., 2010). Additional analysis of a newly identified population of $CXCR5^+$ CD8 T cells, identified in the B cell follicles of mice during acute and chronic LCMV infection, may also have an important role in driving protective immunity (He et al., 2016). These have most recently been associated with spontaneous HIV controllers and produced high levels of IL-21 (Perdomo-Celis et al., 2018).

However, there also appears to be a role for innate cell types in modulating T_{FH} - B cell interactions in chronic viral infection. Activated NK cells have been demonstrated to deplete CD4 T cell responses in the spleen of mice infected with LCMV, resulting in impaired germinal centre formation and LCMV-specific antibody responses (Rydyznski et al., 2015; Waggoner et al., 2011). Follow-up work has since demonstrated that NK cells can directly suppress T_{FH} cells and germinal centre formation by a perforin-dependent mechanism, resulting in fewer antigen mutations within antigen-specific B cells (Rydyznski et al., 2018). Preliminary data from our group, testing the *in vivo* potential of NK cells to regulate vaccine-induced CD8 T cell responses, has shown that NK cell depletion in the setting of chronic HBV infection, increases the frequency and effector function of CD8 T cell responses following therapeutic vaccination, and can result in induction of anti-HBs. In contrast, therapeutic vaccination in the presence of NK cells was insufficient to induce protective humoral immunity (Dr. Mariana Diniz, unpublished data).

6.2.5 Understanding the role of B cells in the liver

The identification of memory B cells in the liver raises the possibility that liver-infiltrating B cells acquire a phenotype adapting them to the tolerogenic environment of the liver. Future studies should seek to pinpoint factors that promote their accumulation, for example the relative contributions of TLR-ligands, inflammatory cytokines and antigenic stimuli. To build on this, it would be of significant interest to examine whether any of these cells are in fact resident within the liver, where they may be uniquely specialised and could represent an attractive target for immunotherapeutic approaches. Recent studies have characterised specialised tissue-resident lymphocyte populations that express an array of receptors mediating their recruitment to, and retention within, tissues. Preliminary investigations using murine parabiosis models has identified a population of resident, influenza-specific memory B cells, that were dependent on germinal centre responses (Allie et al., 2017). These data purport to the existence of tissue-specialised B cells in

mice; however, to date, there has been no investigation of tissue-resident B cell populations in humans, which is currently limited by a lack of residency markers for human B cells.

In contrast, many studies have revealed that a large proportion of CD8 T cells in non-lymphoid tissue are resident (reviewed in Mueller and Mackay, 2016). Our group has recently characterised a population of $CD69^{+}CD103^{+}$ tissue-resident $CD8^{+}$ T cells (T_{RM}) present in the liver but absent in the blood (Pallett et al., 2017). These cells were selectively increased in HBV^{+} patients that controlled infection, and were shown to be specific for all four major HBV proteins. Importantly, these cells appeared to be specialised in line with the liver environment: T_{RM} were capable of rapid production of IL-2 and IFNγ, but showed diminished granzyme production, supporting a role as a non-cytolytic subset involved in immune surveillance. Combined, these data suggested that HBV-specific T_{RM} are located within the liver, where they are uniquely adapted to exert antiviral effector function.

An additional population of liver-resident NK cells, defined as $CXCR6^{+}$T-$bet^{lo}Eomes^{hi}$, have also been identified with unique features adapting them to the liver (Stegmann et al., 2016). These cells produced fewer cytotoxic mediators and pro-inflammatory cytokines, yet upregulated expression of TRAIL – a key ligand in promoting HBV pathogenesis through the deletion of HBV-specific T cells (Peppa et al., 2013), hepatocytes (Dunn et al., 2007) and stellate cells (Singh et al., 2017). Follow-up work, capitalising on HLA-mismatched liver transplants (i.e. where the donors and recipients are HLA-mismatched), suggested that these cells are long-lived and likely did not recirculate (Cuff et al., 2016). Importantly, Cuff et al., set a premise for using explanted HLA-mismatched liver allografts as a method of examining tissue-resident populations. Through methods such as these, it may be possible to identify a unique liver-resident B cell population that may have important roles against hepatic pathogens. Overall, liver-resident lymphocytes are attractive options for therapeutic targets due to their anatomical location and specialised function (Bolte and Rehermann, 2017; Pallett et al., 2017); thus, identification of liver-resident B cells may be an important arm of developing new therapeutic strategies.

In addition, the cytokine-producing potential of B cells is interesting and warrants further investigation, particularly with regard to their ability to produce antiviral cytokines that may have key roles against HBV in the liver. As discussed earlier, effector B cell subsets have been identified that secrete IL-12 and IFN, and amplify T cell responses (referred to as Be-1 cells) (Lund, 2008). In opposition, IL-10 and TGF-β producing B cells have an active role in suppressing immune responses. Data presented in Chapter Four show that peripheral atMBCs have impaired production of antiviral IL-6 and TNFα that was increased following checkpoint blockade. However, the cytokine-producing potential of intrahepatic B cells has yet to be examined and may have important implications in the control of viral replication in the liver. Pro-inflammatory cytokine production is also important to consider with regard to its potential for inducing tissue damage. Thus, it may be

important to examine the bystander effects of B cell-derived cytokines, particularly following checkpoint blockade. Furthermore, previous work from our group has identified an expansion of IL-10-producing B cells with a regulatory phenotype in patients undergoing hepatic flares (Das et al., 2012). In light of our identification of HBsAg-specific B cells and their ability to localise within this liver, it would now be interesting to examine whether antigen-specific cells with regulatory potential, have a role in suppressing intrahepatic immune responses and thus contribute to impaired antiviral immunity at the site of infection.

6.2.6 Neutralising antibody production by HBsAg-specific B cells

The principal way in which B cells contribute to immune control is through the production of antibodies that are able to neutralise infectivity of viral particles and induce killing of infected cells. Despite circulating at comparable levels to HBV-vaccinated or resolved individuals, data from this study suggest that HBsAg-specific B cells are impaired in their ability to produce protective anti-HBs antibody. However, as demonstrated by ELISPOT data showing low-level production of anti-HBs by B cells isolated from patients with CHB and in line with clinical observations of immune complexes in patients with CHB (Gerlich, 2007), patients with CHB are not completely devoid of functional HBsAg-specific B cells, raising prospects for immunotherapeutic boosting of these cells to promote HBsAg seroconversion. This is underscored by observations of HBV reactivation in patients undergoing treatment for B cell lymphoproliferative disorders, showing a role for HBsAg-specific B cells in control of CHB (Evens et al., 2011). Rituximab-mediated depletion of $CD20^+$ B cells in $HBsAg^+$ patients worsened parameters of disease, including: reduction of protective anti-HBs (Tsutsumi et al., 2005); increases in levels of HBV DNA and HBsAg (Viganò et al., 2014); and evolution of hepatitis (Skrabs et al., 2002). Investigation of the quality of antibodies produced by HBsAg-specific B cells was beyond the current capability of this study; however, future studies should now make it a priority to assess ways in which we can recover or replace antibody production by HBsAg-specific B cells. Given that ASCs downregulate surface expression of immunoglobulin following antibody production, direct probe-binding methods for isolating antigen-specific ASCs are of limited used. Instead, the isolation of antigen-specific memory B cells is likely to provide a more viable method. Using a bait to FACs sort single antigen-specific B cells, it is then possible to reconstitute mAbs with corresponding specificity for the BCR of the sorted cell and determine the frequency of mAbs that bind the probe used to sort. This method is currently being employed by multiple groups to develop neutralising antibodies (nAb) for therapeutic use for the treatment of CHB.

The development of nAb as therapies or targets for vaccination has been well-illustrated in HIV infection. Single-cell cloning of memory B cells in HIV-elite controllers facilitated the isolation of antibodies with broad and neutralising activity (Scheid et al., 2009). Injection of highly potent HIV-

specific broadly neutralising antibodies (bnAbs) has demonstrated efficacious protection against HIV against genetically diverse strains in models of simian-HIV (Gautam et al., 2016, 2018) and suppression of viraemia (Barouch et al., 2013; Nishimura et al., 2017; Shingai et al., 2013). Administration of bnAbs together with TLR-7 agonists has since been demonstrated to delay viral rebound following discontinuation of anti-retroviral therapy, suggesting that concurrent innate immune stimulation may represent a potential strategy to target the viral reservoir (Borducchi et al., 2018).

Taking these forward to human trials, monotherapy using an antibody targeting the CD4 binding site of HIV demonstrated potent reduction in viraemia (Caskey et al., 2015). However, monotherapy by itself was insufficient to maintain long-term viral suppression. Recent follow-up work using combinations of bnAbs in the absence of antiretroviral therapy showed that combination therapy could maintain viral suppression and reduced the likelihood of developing resistance (Bar-On et al., 2018; Mendoza et al., 2018). Notably, two individuals studied remained suppressed beyond the presence of the antibody; thus, antibody therapy likely exerts additional effects distinct from direct neutralisation activity.

It is increasingly recognised that antibody-based therapies such as these may have the added advantage of generating or re-activating T cell responses. Hydrodynamic injection of novel nAbs in HBV-transgenic mice increased the frequency of HBcAg- and HBsAg-specific effector CD8 T cells, likely due to the reduction in levels of HBsAg facilitating restoration of exhausted HBV-specific T cell responses (Zhang et al., 2015). However, increasing evidence suggests that antibodies may also augment T cell responses through the formation of immune complexes. Antigen processing and presentation by dendritic cells promotes CD8 T cell activation through a process of cross-presentation. Several studies now suggest that antibody engagement of Fc-receptors on dendritic cells in the form of immune complexes may result in superior priming of T cell responses (Wen et al., 2016). Recent work has suggested that the target of activatory Fc receptors on splenic dendritic cells in the presence of co-stimulatory signals, promotes CD4 and CD8 T cell responses under inflammatory conditions (Lehmann et al., 2017). This enhanced CD4 T cell activation can in turn lead to superior B cell responses, referred to as the *vaccinal effect*, and represents a key hope for antibody therapy in chronic infection and anti-tumour treatment (DiLillo and Ravetch, 2015).

Overall, through studying the phenotype of B cells producing functional antibodies in patients that naturally resolve infection, it may be possible to identify targets by which to promote the natural production of nAb in CHB. In HBV, two classes of nAbs have been described that target the viral entry receptor, NTCP, and HSPGs (outlined in 1.6.1). The neutralising efficacy of anti-HBV antibodies has been well demonstrated in primate models: inoculation of chimpanzees with human monoclonal antibodies prior to viral challenge prevented the development of infection (Hong et al.,

2004; Kim et al., 2008). Of note, the antigen incorporated within our HBsAg-bait reagent did not span the Pre-S1 region and therefore may not detect B cells responsible for secreting antibodies that block viral binding to the hepatocyte (Sankhyan et al., 2016). Thus, future studies should also aim to dissect antibody responses against different HBsAg epitopes. Comparison of the BCR repertoire of HBsAg-specific B cells to anti-HBs secreted by HBV-resolved patients could also provide valuable insights into the gold-standard antibody or B cell phenotype required for neutralisation of HBV.

6.3 Outlook: paving the way for B cell-targeted research in HBV

To date, none of the currently developed antiviral strategies, including therapeutic vaccination, viral entry inhibitors or immune modulators, reliably achieve functional cure. Looking forward, it is likely that attempts to promote clearance of HBsAg may reduce HBsAg-induced tolerance enough for the host to re-establish protective immune responses. Using HBV-infected murine models, with contrasting high and low levels of circulating HBsAg, Zhu et al., showed that clearance of HBsAg via therapeutic anti-HBs neutralising antibodies effectively restored protective CD4 T cell and B cell responses to prophylactic HBV vaccination, ultimately leading to anti-HBs seroconversion (Zhu et al., 2016). In this way, immunotherapeutic approaches to enhance cytotoxic potential of CD8 T cells to clear infected hepatocytes and reduce the circulating viral/antigen load may have knock-on effects on lifting dysfunction of B cells, whilst simultaneously decreasing the occurrence of immune complex formation and releasing the effector function of un-complexed antibodies. However, the identification of a number of defects within HBsAg-specific B cells themselves identifies novel targets for immunotherapeutic approaches aiming to boost the production of neutralising antibodies and promote loss of HBsAg – regarded as the hallmark of functional cure. These include targeting of inhibitory receptors and the potential for agonistic anti-CD40 monoclonal antibodies in promoting antibody responses. This study also highlights the need to investigate knock-on effects of immunotherapeutic approaches on B cell function in terms of promoting pro-inflammatory cytokine production and potentiating dysfunctional B cell subsets.

This study has been closely supported by a back-to-back paper that also showed defective antibody production by HBsAg-specific B cells in patients with CHB, partially rescued through PD-1 blockade (Burton et al., 2018; Salimzadeh et al., 2018). During the writing of this thesis, a further paper has been published that again demonstrates decreased anti-HBs production by B cells from patients with CHB, alongside an expansion of B cells with an atMBC phenotype (Poonia et al., 2018). Notably, the study from Poonia et al. reinforces the link between the accumulation of atMBCs and defective antibody production, by demonstrating impaired anti-HBs production by atMBCs relative to cMBCs. Finally, the authors show that culture of B cells with HBV viral proteins can directly induce an upregulation of inhibitory receptors on B cells, lending support to the concept that chronic antigen exposure impairs B cell responses in HBV.

Overall, this study has identified previously unappreciated roles for B cells in the immune dysfunction observed in CHB, including decreased antiviral cytokine production and secretion of anti-HBs (reviewed in Neumann-Haefelin and Thimme, 2018). Importantly, these results make a strong argument for B cell dysfunction, as opposed to HBsAg-specific B cell depletion, as the main reason for the lack of anti-HBs in CHB. The description of the phenotype of both HBsAg-specific B cells and atMBCs raises multiple new avenues of research into possible mechanisms underpinning B cell dysfunction in chronic viral infections. Indeed, many open questions remain

including the cellular and molecular mechanisms leading to HBsAg-specific B cell dysfunction, as well as the optimal strategy to restore anti-HBs responses on the way to achieving functional cure. Figure 6.1 summarises the key areas of interest highlighted by this study and contextualises these within what is already understood surrounding adaptive immune dysfunction in CHB. Overall, this study provides a precedent for B cell-targeted research in hepatitis B.

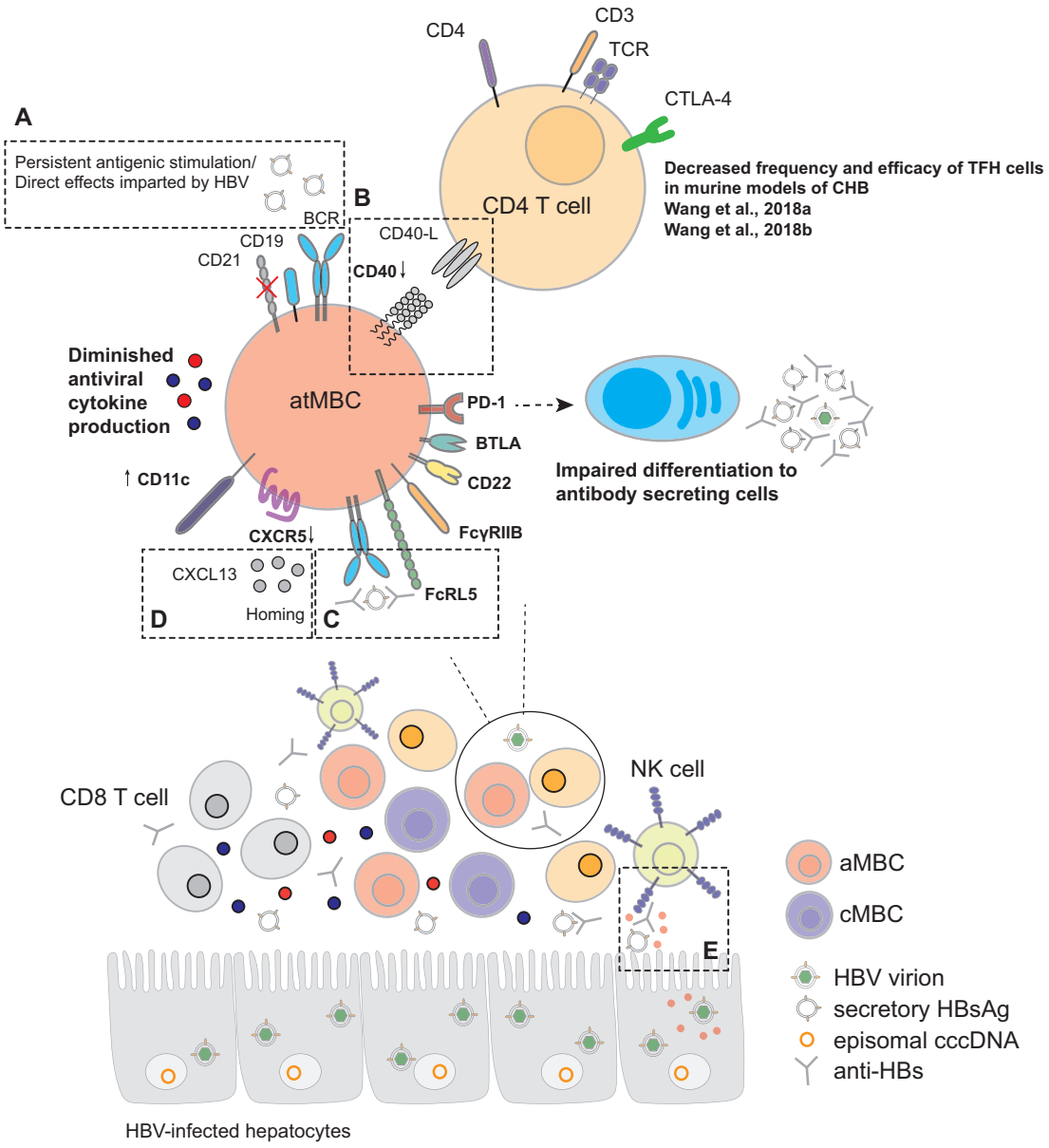

Figure 6-1 Conceptualisation of the role of atMBCs in immune dysfunction in CHB

Features identified directly within this thesis are highlighted in bold. Questions arising for future research discussed within this thesis are demarcated by boxes. **A**. How does persistent stimulation/the presence of HBV drive B cell dysfunction? **B**. Are B cell – CD4 T cell interactions impaired in HBV, and if so, does this impact the ability to form a functional humoral response? **C**. Can circulating HBsAg:anti-HBs immune complexes further contribute to the suppression of atMBC responses? **D**. Is the homing of atMBCs/HBsAg-specific B cells to lymphoid organs impaired, and does this have an effect on the production of high-affinity antibody? **E**. Can local production of anti-HBs have a role in killing infected hepatocytes through ADCC?

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